A STUDY OF ACETYLCHOLINESTERASE AND CHOLINESTERASE IN THE FETAL MOUSE BRAIN AND A STUDY OF THE EFFECTS OF THREE TERATOGENS, VITAMIN A, CYCLOPHOSPHAMIDE AND SODIUM VALPROATE ON THE FETAL MOUSE CENTRAL NERVOUS SYSTEM

by

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ABSTRACT

There were two aims in this thesis. Firstly, to investigate cholinesterase and its isoenzymes in the fetal mouse brain, and secondly to study drug-induced fetal damage with the following objectives in mind: (i) to examine new markers for the evaluation and prediction of the teratogenic potential of drugs, and (ii) to try and throw more light on pathogenic mechanisms of drug injury with particular reference to the developing fetal central nervous system.

Acetylcholinesterase activity in brain homogenates was determined colorimetrically and the isoenzymes were separated by polyacrylamide gel electrophoresis. A cyanmethaemoglobin method was used to measure the contribution of acetylcholinesterase activity in blood to total brain esterase activity. Cholinesterase activity was estimated colorimetrically, and with the aid of enzyme inhibitors and polyacrylamide gel electrophoresis.

The effects of three central nervous system teratogens, vitamin A, cyclophosphamide and sodium valproate when administered during embryonic development, on gross fetal parameters in C3H mice including embryolethality, gross morphological abnormalities, fetal weight, brain weight, brain acetylcholinesterase and its isoenzymes, and in some instances brain total protein content and choline acetyltransferase activity, were assessed. Preliminary studies were also performed with a view to future areas of research on (i) the effects of vitamin A when administered during the pre-implantation period on viability/esterase enzyme activity, cell number, mitotic index and chromosome structure in the 81h blastocyst; (ii) the influence of vitamin A on C3H fetal mouse brain proteins using high resolution two-dimensional electrophoresis; and (iii) the effects of cyclophosphamide and vitamin A on cephalic DNA damage utilising a DNA unwinding assay to detect DNA strand breaks.

Substantial acetylcholinesterase activity of ± 3nmol/min/mg was present in 17-day to 19-day fetal mouse brains and 5 isoenzymes were present on electrophoresis. The contribution of acetylcholinesterase activity in blood was low at approximately 3%. Similarly, fetal mouse brain cholinesterase activity was found to be very low and the effect of teratogens on this enzyme was not assessed.

A rise in the incidence of malformations and embryolethality with increase in dose, and after administration earlier in gestation occurred with all three teratogens. A growth-inhibitory effect was another feature although this was most pronounced after cyclophosphamide administration. Acetylcholinesterase activity was affected by the teratogen used and its time of administration as well as other factors such as growth inhibition, haemorrhage and repair processes. Vitamin A administration on day 10 of gestation was associated with a greater acetylcholinesterase activity compared with controls, which was not accompanied by a change in brain total protein content or choline actyltransferase activity. Cyclophosphamide and sodium valproate administration during the embryonic period were associated with a lower acetylcholinesterase activity in near-term fetuses. However, in fetuses examined two days after cyclophosphamide administration there was a greater acetylcholinesterase activity associated with an increase in haemoglobin and a decrease in choline acetyltransferase. A higher acetylcholinesterase activity was also observed in exencephalic brains. Vitamin A administration was associated with a higher activity of isoenzyme 5 whereas cyclophosphamide and sodium valproate administration resulted in a lower peak height for band 4.

When vitamin A was administered during the pre-implantation period 60h after copulation no effect on viability/esterase enzyme activity, cell number, mitotic index or chromosome structure was observed in 81h embryos. However, a striking incidence of abnormalities was noted in fetuses examined

near term.

This study suggested that teratogenic doses of vitamin A modified the brain protein pattern of the fetal mouse with a possible broad spectrum deletion of protein spots and the appearance of a limited number of new spots.

There was no evidence of DNA strand breaks induced by vitamin A, which contrasted with obvious cephalic DNA damage after cyclophosphamide administration.

The potential of these techniques in the prediction of the embryotoxicity of drugs, and progress in the understanding of underlying mechanisms are discussed.

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CHAPTER I

TERATOLOGY AND THE EFFECTS OF VITAMIN A, CYCLOPHOSPHAMIDE AND SODIUM VALPROATE ON THE FETUS

1.1. INTRODUCTION TO TERATOLOGY

Currently, a teratogen is considered to be an agent that induces structural malformations, metabolic or physiological dysfunction, or psychological or behavioural alterations or deficits in the offspring (Schardien, 1985). Chemicals capable of inducing malformations also have the potential for inducing other deviations of development, which are referred to as embryotoxic effects and fit under the umbrella of the term "developmental toxicity". In addition to malformation other manifestations of toxicity are growth retardation, death and functional alteration.

Whether or not a given chemical has the potential to induce congenital abnormalities is governed by established fundamental principles of teratogenesis, largely formulated by Wilson (Wilson 1965, 1973): susceptibility depends on the species, the developmental stage at the time of exposure, the dosage of the agent, the nature of the agent used and the physiological or pathological state of the mother.

Not all species are equally susceptible to teratogenic influence of a given chemical (Schardien, 1976 : 20). This is partly explicable on genetic differences (Wilson, 1977 : 49). The reaction to a specifice teratogen may be due to differences in a species rate of metabolism, as well as to qualitative differences in metabolic pathways (Burns and Conney, 1964). Accessibility of a drug to the embryo, dependent on placental differences, may also account for some differences in species susceptibility. As noted by Kalter (1968), inter- and intra-species variability

may be manifested in several ways: (i) An agent that is teratogenic in some species may have little or no effect in others. For example, man and higher primates are extremely sensitive to thalidomide, whereas most other mammals are quite resistent. (ii) A teratogen may produce similar defects in various species, but these vary in frequency. (iii) A teratogen may induce certain abnormalities in one species that are entirely different from those induced in other species.

The developmental stage at the time of exposure is crucial. During the pre-differentiation period in early gestation the embryo is remarkably resistant to teratogenesis (Austin, 1973; Spielman et al., 1977). It has been generally accepted by many teratologists that exposure during this period results in either death of the embryo before implantation or survival to term without any abnormality - the all-or-none phenomenon (Wilson, 1977 : 51; Tuchmann du Plessis, 1975 : 40). Austin (1973) has suggested that the reason for this resistance to teratogenesis is that the undifferentiated cells of the early embryo are totipotent and possess the capacity to form any part of the future individual, so that losses of cells can to some extent be accounted for. The effect on the cleavage embryo depends on the number of cells killed or inhibited; above a certain proportion the embryo dies, below that figure the remaining cells multiply to replace those lost and subsequent development is essentially normal.

The response to a teratogenic agent, however, is more complex than an all-or-none phenomenon. Spielman et al., (1977) showed that whilst cyclophosphamide administered before implantation had no effect on implantation of rat embryos and did not cause malformations, the drug caused a decrease in cell number of blastocysts, subsequent fetal growth retardation and a dose dependant increase in resorption rate with the majority of fetuses dying during the organogenesis period. Kola (1985) has also shown that viable cyclophosphamide treated embryos have dose related structural

and functional deficits. Iannaccone (1984) demonstrated histological and chromosomal abnormalities in the absence of structural malformations at term after exposure of blastocysts to methylnitrosurea, and there are also some reports of malformations resulting from exposure to teratogens during the preimplantation period (Rugh and Grupp, 1959; Gottschewski, 1964; Moriss, 1972; Takeuchi, 1984). Klein and Pierro (1983) have suggested that exposure of preimplantation embryos to noxious agents may manifest as behavioural abnormalities.

During the embryonic stage embryological differentiation and organogenesis occur. During the "critical period of organogenesis" there is particular vulnerability for induction of structural defects to organs and systems. As the more basic organisational patterns of organ formation are completed, the likelihood of major structural deviation naturally diminishes (Schardien, 1976 : 17; Wilson, 1977 : 52). The embryonic stage is followed by fetogenesis which is characterised by growth and histological differentiation and continues for a variable period after birth. Because the central nervous system is continuing its complex development during this stage abnormalities can be produced, typically histological or functional alterations.

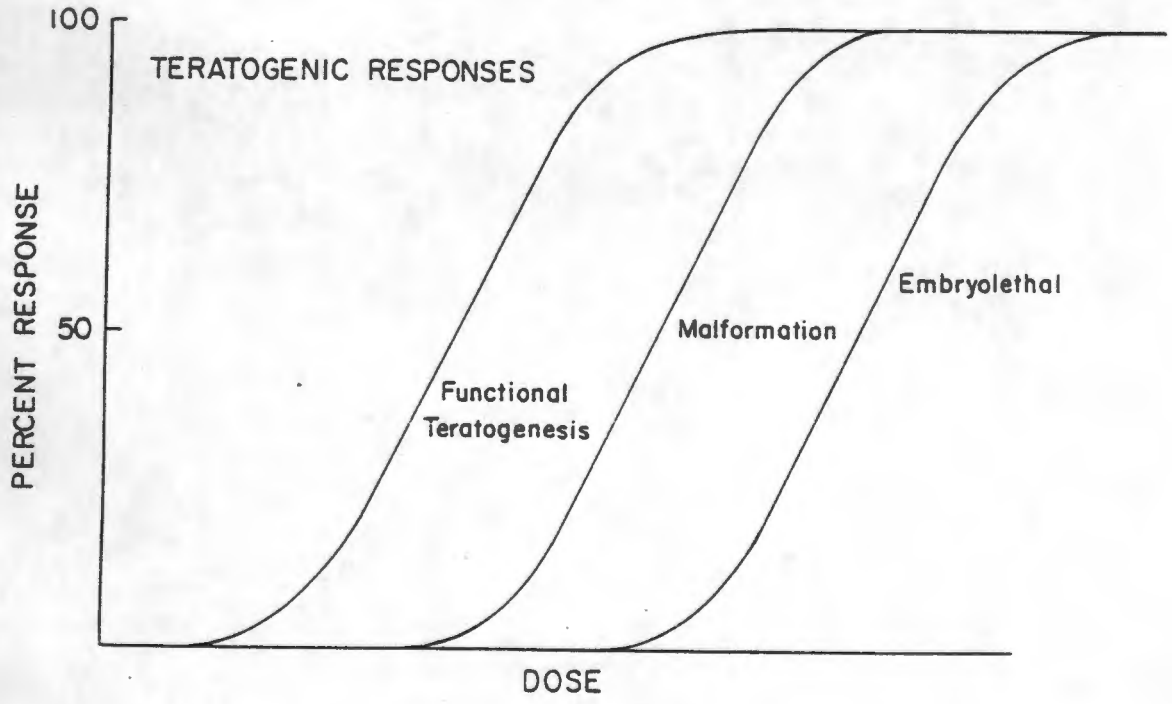
Teratogenicity is governed by dose-effect relationships. The dosage of a given teratogen lies within a narrow zone between that which will have no discernible effect and that which causes malformations or death. Typically the dose response curve has a steep slope, sometimes going from minimal to maximal effect levels with doubling the dose (Wilson, 1973). An exception, however, is thalidomide which is teratogenic at low doses but can be tolerated without embryolethality at several multiples of this dose . Functional defect, malformation and embryolethality are thought to have their own dose-response curves, the curve for functional

defect to the left and that for embryolethality to the right of the malformation curve (Snell, 1982a, Fig. 1.1.). A fourth curve representing offspring growth was thought by Snell to intervene probably between the functional and malformation curves, although he stated that it was not entirely clear whether for some agents it would prove to be isomorphic with or even to the left of the functional defects curve.

The current concept concerning dosage is that teratogenic chemicals are hazardous only when they disrupt development of the conceptus at dosages not toxic to the adult (Johnson, 1980, 1981). The implication is that a chemical producing terata is not necessarily a teratogenic hazard because the chemical would be in essence regulated solely by the toxicity to the adult animal. It is well known that, due to particular vulnerability of certain embryonic cells (Neubert et al., 1971), the embryo usually has a greater susceptibility to chemicals than does the adult.

Fig. 1.1.

THEORETICAL SET OF DOSE-RESPONSE CURVES SHOWING THE RELATIONSHIP BETWEEN THE THREE PRIMARY MANIFESTATIONS OF TERATOGENESIS



The duration of chemical treatment is another variable. In general acute dosage schedules are of greater teratogenic insult to the developing embryo than chronic dosage schedules (Schardien, 1985:10). Similarly, differences may exist in teratogenic response when a chemical is given by different routes of administration or in different dosage forms. With vitamin A, oily solutions must be used orally to produce teratogenic effects, whereas parenterally only aqueous solutions are effective (Kalter, 1968b).

The nature of the agent determines its access to the embryo (Wilson, 1977: 59). Placental transfer is moderated by the drug's lipid solubility, molecular weight, degree of ionisation and protein binding and by placental properties such as maternal and fetal blood flow, drug metabolism and placental age.

Finally, it is the interaction of fetal, maternal and environmental factors that determine the outcome of a particular teratogenic insult. Maternal age, parity, weight, basal metabolic rate, size and constitution of placenta and hormone states are all variables in strain susceptibility (Tuchmann du Plessis, 1975:45). These are subject to modification by environmental factors such as diet, temperature and circadian and seasonal effects (Kalter, 1965; Woollam and Millen, 1960; Sauerbier, 1981).

The final manifestations of abnormal development may be malformations, death, growth retardation or functional disorder. The specific types of malformations occurring in different species are variable. While most are observed in nearly all species, the individual malformations and their frequency are somewhat species-dependent. Thus eye defects, exencephaly, polydactyly and cleft palate are seen quite commonly in mice (Flynn, 1968; Kalter, 1968a). It is important to recognise that spontaneous malformations may occur, and this varies among different species. For C3H mice

the rate is 3,3% (Heinecke, 1972).

As an indicator of developmental toxicity fetal size is an important parameter in the assessment of potential teratogens. As a rule any potent teratogen will produce growth retardation in addition to malformation and resorption (Brent and Jensh, 1967). Indeed, overall growth retardation is thought by some investigators to constitute a state of increased susceptibility to congenital malformations (Spiers, 1982).

Intra-uterine death, like other developmental toxicity is distributed along a dose-response curve. When there is undue toxicity early in pregnancy the embryo dies and is resorbed. If death occurs later in pregnancy the fetus cannot be wholly resorbed and a stillborn or dead and often macerated fetus is the result. Mortality may be due primarily to the direct action of chemicals on the conceptus regardless of whether or not it is malformed, or it may be secondary to maternal effects (Kalter, 1980). Although intrauterine death is easy to quantitate,its relationship to other toxicity varies (Wilson, 1980).

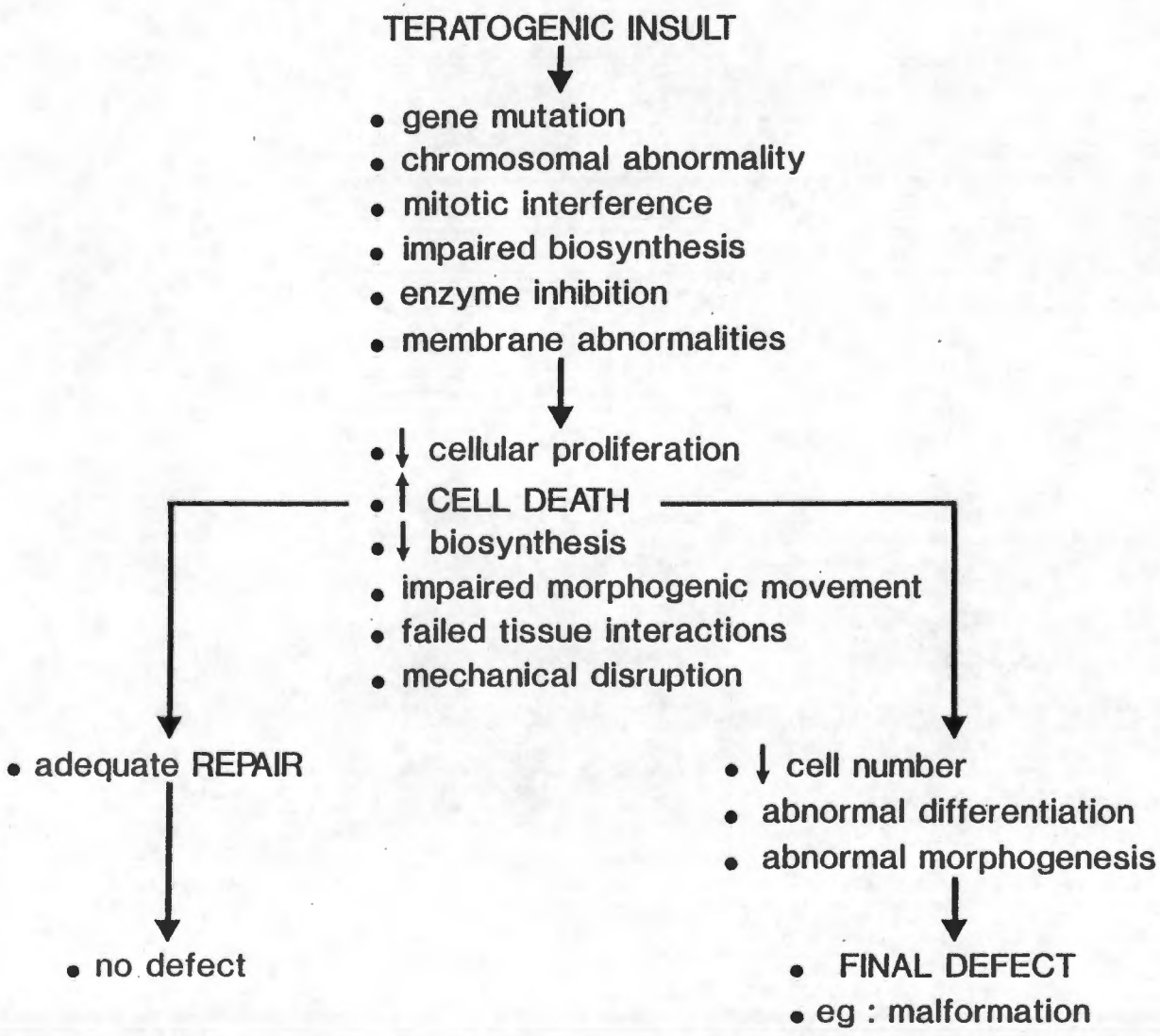
Functional impairment resulting in lower intelligence or impared behaviour may be the result of more subtle, non structural alterations produced by teratogens. Agents that are teratogens of the central nervous system are also behavioural teratogens when given at dosages below which major malformations are produced (Vorhees et al.,1979, Mooney et al.,1981). It has been suggested that such alterations in behaviour arise as a result of drug-induced modification of development of specific neuro-transmitter systems (Leonard, 1981). Apart from behavioural studies it has been proposed that evaluation of perinatal enzymology represents an approach for improved testing of postnatal functional development because quantifiable deviations in enzyme activity may be sensitive indicators for deleterious effects (Andrew and Lytz, 1981)

At present there are no firm or unequivocal explanations of the mechanisms of action of any teratogenic agent. The best overview of the mechanisms by which teratogens induce abnormal development is probably still that given by Wilson (1977). The possible mechanisms include mutation, chromosomal aberration, mitotic interference, altered nucleic acid or energy sources, enzyme inhibition, osmolar imbalance or altered membrane characteristics. These are ultimately manifested as one or more types of abnormal embryogenesis, such as cell death, reduced biosynthesis, impaired morphogenetic movement, failed tissue interaction, or mechanical disruption. The embryo is particularly vulnerable to alteration of normal biosynthesis (including DNA, RNA and protein biosynthesis) because it must maintain critical developmental schedules and because the cells are undergoing rapid proliferation (Ritter, 1977). This vulnerability may lead to decreased cellular proliferation or cell death. Whatever the basic mechanism many teratogens cause cell death and a reduced proliferative rate. The lowered cell number which results from these effects appears to play an important role in the genesis of malformations (Scott, 1977). The primary result of cell death is thought to be aberrant differentiation. Many complex processes are essential for normal differentiation, and many of these occur in parallel. These include cell-cell interaction, cytodifferentiation, morphogenesis, growth, and expressivity of the embryonic genome. The cell surface plays a central role in these events (Skalko, 1981). Aberrant differentiation ultimately leads to malformation (Schreiner and Holden, 1983).

However, embryonic tissue has a tremendous capacity for repair following injury. If the regeneration is adequate and "in time", no malformations will be seen (Ritter, 1977).

I have depicted the current status of teratogenic mechanisms and the pathogenesis of malformations in Fig. 1.2.

Fig. 1.2. TERATOGENIC MECHANISMS AND THE PATHOGENESIS OF DEVELOPMENTAL DEFECTS



In seeking new markers of teratogenic potential of drugs the brain is a useful target organ to study because its complex development continues throughout gestation and CNS anomalies are the most common and most important of the congenital defects. The high frequency of occurrence of CNS anomalies is a result of the complexity of its development (Ornoy and Yanai, 1980). Most anomalies are minute and morphological changes may be difficult to elucidate by present conventional techniques. More sensitive methods, such as detecting neuro-chemical changes, are therefore needed.

1.2. VITAMIN A AS A TERATOGEN

The teratogenic potential of large doses of vitamin A (retinol) in animals has been recognised for over 30 years. Cohlan, (1953; 1954) induced a variety of malformations in Wistar rats such as exencephaly, hydrocephalus, spina bifida, cleft palate and eye defects, using a daily dose of 75 000 IU, equivalent, weight for weight, to more than 25 million IU for an adult human. Hundreds of animal studies have since confirmed the teratogenicity of vitamin A, well reviewed by Geelan (1979). Of particular importance is the recent focus of interest on the vitamin and its analogues in man because of a number of reports of birth defects (Rosa et al., 1984; Lancet editorial, 1985). There are at least 18 reports of suspected vitamin A induced human birth defects, which include abnormalities of the nervous system, cranio-facial, cardio-aortic and genitourinary malformations (Gal et al., 1972; Bernhardt and Dorsey, 1974; Mounoud et al., 1975; Stange et al., 1978, Rosa, Wilk and Kelsey, 1986). Of note is that doses of vitamin A as low as 15 000 to 60 000 IU daily, which are comparable to teratogenic doses in animals, have resulted in abnormalities.

Of further relevance is the fact that there have been at least 44 birth defect case reports associated with the maternal use of vitamin A analogues

or the retinoic acid derivitives, isotretinoin and etretinate. Typically, absent or small low external ears or canals, central nervous system abnormalities and/or cardioaortic defects were present (Rosa, 1984; Rosa et al., 1984; Rosa, Wilk and Kelsey, 1986).

Whilst the relevance to humans stimulated my interest in this teratogen, of particular pertinance to my studies was the fact that vitamin A is a well established central nervous system (CNS) teratogen. Exencephaly, described in the first studies with vitamin A in the rat (Cohlan, 1953; 1954) is a common abnormality which also occurs, albeit with a lower incidence, in the mouse (Giroud and Martinet, 1959; Kalter and Warkany, 1961-Murakami and Kameyama, 1965; Theodosis and Fraser, 1973), and is due to nonclosure of the rostral neural plate. Minor closure defects of the neural tube may result in meningocele or meningoencephalocele (Giroud, Martinet and Solere, 1958; Geelan, 1979), while abnormal closure of the spinal cord which occurs less commonly, results in spina bifida.

Other abnormalities include hydrocephalus, which does not occur frequently with vitamin A treatment (Murakami and Kamayama, 1965), microcephaly (Kocher, 1967) and "crowded brain", where the brain appears too large for the cranium with no sign of hydrocephalus (Kalter and Warkany, 1961).

In the mouse most abnormalities of the CNS have been produced by dosing on one or more days between 8 and 10 days post conception. However, the brain continues to undergo differentiation and proliferation during pregnancy. Abnormal neuroblasts were noted in the cerebral cortex of Swiss-Webster mice treated in the second half of pregnancy on days 15, 16 and 17 (Langman and Welch, 1967). Other histological evidence of an embryotoxic effect on the CNS has been provided during the embryonic phase. Geelen, Langman and Lowdon (1980) found swelling and degeneration of neuroepithelial cells with large intercellular spaces in mouse embryos treated

with the vitamin just before brain vesicle closure. Similar changes in the mouse neuro-epithelium were noted by Theodosis and Fraser (1978). A feature of these studies was the rapid onset of action of vitamin A where histological changes were apparent within 2 - 4 hours of treatment.

Further evidence that vitamin A is a CNS teratogen is provided by the observations that it can produce behavioural abnormalities (Butcher et al., 1972; Vorhees et al., 1978; Vorhees et al., 1979; Mooney et al., 1981). Of relevance is that these behavioural effects can be produced at doses just below the teratogenic threshold for the induction of morphological abnormalities. Also, behavioural abnormalities in offspring can be produced with prenatal exposure to larger doses after the period of organogenesis (Hutchings et al., 1973, 1974).

As with any teratogen the types and incidence of malformations depend on the stage of pregnancy during which vitamin A is administered, on the dose, and to a lesser extent on the species and strain. Many investigators have used multiple treatments of vitamin A, although a single administration is just as effective if time and dose are chosen carefully (Shenefelt, 1972). The effects of single doses of 10 000 IU vitamin A administered on different days after conception in female mice of the A/J, DBA/iJ and C3H/J inbred strains were studied by Kalter and Warkany (1961).

Treatment of females between 7 and 8 days post conception caused all but one of the young conceived to be resorbed, whereas a single administration of vitamin A between 8 and 9 days after conception resulted in a 58% resorption rate, and a widespread and complex syndrome of malformations in the fetuses. Ten thousand IU vitamin A between 9 and 10 days post conception resulted in a 30% resorption rate, with only a part of the above syndrome in the fetuses. Both groups had defects of the head, face and mouth, but those treated between 9 and 10 days after conception had more severe abnor-

malities of these parts than those treated earlier. A frequent defect of the head was exophthalmos of various degrees, often with absence of the eyelids.

An agent can have an embryotoxic effect by affecting the maternal organism, the placenta or the embryo itself. In vitro studies, where embryos are cultured in the presence of excess vitamin A, have demonstrated that the teratogenic effects of vitamin A are due to a direct effect on the fetus (Geelan, 1979; Morriss, 1972; Morriss and Steele, 1974; Morriss and Steele, 1977; Kochar, 1975; Steele et al., 1982. In vivo studies have shown that the vitamin reaches the embryo. ^{3}H-vitamin A acid administered to pregnant mice demonstrated radioactivity in the embryo one hour after dosing (Kochar and Kwasigroch, 1975; Kochar, 1976). Furthermore, a teratogenic dose produced a value in the embryo which was 26-fold the value measured after administration of a non-teratogenic dose.

Since very low concentrations of vitamin A are teratogenic in vitro, it appears that only very small amounts of vitamin A reach the embryo in vivo. This may be related to factors involved in maternal vitamin A storage, transport and metabolism (Morriss and Steele, 1974). The liver has an enormous storage capacity for vitamin A and the vitamin is transported in plasma bound to retinol-binding protein which renders it inactive (Morriss and Thomson, 1974; Dingle, Fell and Goodman, 1972).

The mechanisms by which the teratogenic effects of vitamin A are produced remain unclear despite numerous studies.

Vitamin A has a toxic effect on cell membranes. Lipoprotein membranes have been shown to exhibit increased permeability and decreased stability

in the presence of excessive vitamin A (Lucy et al., 1963). Because of its amphipathic molecular structure retinol is highly surface active. It is able to penetrate rapidly into lipid monolayers and hence into cell membranes (Bangham et al.,1964). The damaging action of large doses of vitamin A may then result when, following interaction between membrane phospholipids and retinol molecules, oxidation of the double bonds of retinol and co-oxidation of membrane phospholipids occur (Dingle and Lucy, 1965). Vitamin A has also been reported to have an enzyme mediated effect on membrane microviscosity (Meeks et al., 1981).

It has been shown to haemolyse erythrocytes and is able to disintegrate cell and nuclear membranes of fibroblasts and cause mitochondria to swell (Lucy et al., 1963). The increased volume of fetal mouse neuroblasts after maternal exposure to large doses of vitamin A was speculated to be due to increased permeability of lipoprotein membranes within the neuroblasts and to swelling of mitochondria (Nawar et al., 1979). Chepenik and Waldman (1983) suggest that an agent such as vitamin A, which alters membrane lipid metabolism, can result in abnormal differentiation, which ultimately accounts for birth defects. (It has been postulated that most fetal malformations are attributable to aberrant differentiation (Schreiner and Holden, 1983)).

Vitamin A has been shown to interefere with mitosis in epithelial tissues in vitro (Aydelotte, 1963a, b) and in neuroepithelial cells of fetal mouse cerebral cortex after maternal treatment (Langman and Welch, 1967). In the latter experiment vitamin A had an inhibitory effect both on mitosis and DNA synthesis, prolonging the cell cycle by approximately 40% of its normal value, and affected the differentiation of neuroblasts. The vitamin has also been found to markedly inhibit cell proliferation (assumed from a decreased rate of thymidine uptake) (Kochar, 1968), and causes growth retardation and inhibition of differentiation in rat embryos in vitro (Morriss

and Steele, 1974), and inhibits chondrogenic differentiation of embryonic mouse mesenchymal cells *in vitro* (Zimmerman and Tsambaos, 1985). Other workers have noted that vitamin A decreases *DNA synthesis* (Dingle, Lucy and Fell, 1961; Nanda, 1971; Morriss, 1972; Kochar, 1975).

The hypothesis can therefore be made that excess vitamin A interferes with DNA synthesis, and either secondarily or independently, inhibits mitosis, cell multiplication, growth and differentiation, which leads to abnormal morphological development. It appears unlikely, however, that reduction in DNA synthesis is the primary mechanism. Kochar (1975) noted that concentrations of vitamin A ten times that which cause malformations in culture did not alter DNA synthesis, and Eckhert and Hurley (1979) were unable to show an effect on DNA synthesis in rat fetuses exposed to doses of vitamin A which had induced a 61% incidence of malformations. It is more likely that vitamin A inhibits cell proliferation, mitosis or differentiation by a different mechanism, and this leads to abnormal development.

Maternal administration of large doses of vitamin A in rats and mice has been shown to produce changes in the acid-*mucopolysaccharide metabolism* of embryonic tissues, associated with morphological changes (Kochar and Johnson, 1965; Kochar *et al.*, 1968). This has been suggested as a clue to the mechanism of origin of some malformations such as cleft palate (Kochar and Johnson, 1965).

Vitamin A has been found to increase *prostaglandin synthesis* (Levine and Ohuchi, 1978). Mouse embryo palate mesenchyme cells (Chepenik and Greene, 1981) and chick embryo limb bud mesenchyme cells are able to synthesise prostaglandins and Chepenik and Waldman (1983) speculate that an effect on prostaglandin synthesis may be a mechanism by which vitamin A could alter differentiation.

It is apparent from the literature as outlined above that a number of different teratogenic mechanisms have been postulated for vitamin A. It is not known which of these, if any, are most important. To what extent these are primary mechanisms or are secondary to a more fundamental embryotoxic effect is not clear. It has recently been postulated that some fundamental molecular step in the process of differentiation provides a target for the action of retinoic acid, a vitamin A analogue (Kochar, 1985). Sporn and Roberts (1983) have suggested that to be compatible with the wide ranging effects of retinoids documented so far, any hypothesis put forward for their molecular mechanism of action must include a role in gene expression.

Vitamin A was chosen as a suitable teratogen firstly, because it is a well established central nervous system teratogen and therefore likely to be an appropriate probe for investigating the effects on brain biochemical markers such as acetylcholinesterase; secondly, because of its relevance to human birth defects, which are similar to those produced in animals; and thirdly, to try and throw more light on the mechanisms of its teratogenic action, since it is apparent that these have yet to be adequately elucidated.

1.3. CYCLOPHOSPHAMIDE AS A TERATOGEN

Cyclophosphamide, an alkylating agent, has a growth inhibitory action, produces chromosomal aberrations, is mutagenic and has embryotoxic and teratogenic effects in several animal species (Tuchmann du Plessis, 1975 : 89). It is one of the best studied teratogens, which has been recently reviewed by Mirkes (1985), and produces anomalies primarily of the central nervous system and skeleton in the mouse. Four reports have appeared in the literature suggesting a correlation between maternal exposure to cyclophosphamide and appearance of birth defects in humans (Greenberg and Tanaka, 1964; Toledo et al., 1971; Sweet and Kinzie, 1976).

In mice, administration of the teratogen is associated with a dose-dependent decrease in fetal growth, as evidenced by a decrease in fetal weight and crown-rump length, and a dose dependent increase in embryo-lethality (Gibson and Becker, 1968; Ujh'azy et al., 1979). Malformations caused by cyclophosphamide include exencephaly, hydrocephalus, encephalo-coeles, microcephaly, exophthalmos, stunted or crooked tails, skeletal defects, cleft palate and aphakia (Ujh'azy et al., 1979; Gebhardt, 1970; Gibson and Becker, 1967; 1968). Extensive haemorrhage and necrosis are regularly associated with the embryotoxic effects of cyclophosphamide (Padmannabhan and Singh, 1983). Single administrations of cyclophosphamide are effective and malformations depend on the day of exposure to the tera-togen. The most sensitive period of exposure for producing murine malfor-mations is from day 10 through day 12 of pregnancy (Gibson and Becker, 1968). The dose that is embryotoxic appears to vary with different strains of mice. For example, in Swiss-Webster mice a dosage range of 5-10mg/kg cyclophospha-mide resulted in weight reduction or increased in-utero mortality, and 20mg/kg was associated with developmental abnormalities (Gibson and Becker, 1968). In contrast, with the DM/MK strain, 25mg/kg was ineffective, and higher doses of 50-100mg/kg were needed to produce teratogenic effects (Shoji and Ohzu, 1965). Both subcutaneous and intra-peritoneal administra-tion have been found to be suitable for producing embryotoxic effects.

Histological evidence of the central nervous system effects of cyclophos-phamide has been published by von Kreybig and Schmidt (1967) and Wendler (1979), who demonstrated dose dependent massive cell necrosis and degenera-tion with oedema and haemorrhage. This involved the whole brain during embryogenesis, but in the early fetal stage the neuroepithelial and mantle layer were particularly involved. In the chick embryo cerebellum, degeneration of Purkinje cells, retarded development and disorganisation of morphogenetic events have been documented (Singh et al., 1973).

The onset of necrosis induced by cyclophosphamide appears to be a rapid process. Wendler (1979) noted massive cell destruction in the fetal rat brain 24h after administration. In embryonic limb buds evidence of excess necrosis was apparent 5h after exposure and by 24h massive necrosis was observed (Manson et al., 1982). Repair has been recorded in the course of the second 24h after administration in the fetal rat brain by von Kreybig and Schmidt (1967), but if an embryolethal dose of cyclophosphamide was used, resulting in fetal death after 2-3 days, repair did not occur. Wendler (1979) also noted evidence of repair and regeneration in fetal rat brains 72h after application of cyclophosphamide. In experiments with 5-fluorodeoxyuridine, a compound which blocks DNA replication leading to degeneration and necrosis of neuroepithelial cells in the neocortex, evidence of repair was striking as early as 24h after application, and at 72h the neocortoex appeared normal (Langman et al., 1980).

Behavioural studies have provided further evidence of the central nervous system toxicity of cyclophosphamide. Rodier et al., (1974) showed that prenatal drug treatment resulted in learning defects and abnormal behaviour. Clyclophosphamide, administrered to newborn mice also produced functional impairment, and some of these defects were independent of gross body malformations (Preache and Gibson, 1976).

Cyclophosphamide is a proteragen and must be activated in vivo by hepatic cytochrome P-450 oxidation to cytotoxic alkylating metabolites (Fantel et al., 1979; Kitchin et al., 1981; Greenaway et al., 1982; Hales, 1983). Numerous studies have been performed to determine which of the metabolites produced act as the ultimate teratogens. Phosphoramide mustard has been shown to be an important teratogenic metabolite (Mirkes et al., 1981; Spielmann and Jacob-Muller, 1981; Nau et al., 1982). Although some studies failed to demonstrate that a second metabolite, acrolein was teratogenic

(Mirkes et al., 1981; Schmid et al., 1981), Hales (1982) demonstrated by intra-amniotic injection that acrolein was teratogenic over a narrow dosage range. These results were confirmed by Mirkes et al., 1984.

Although the mechanism of cyclophosphamide teratogenicity is unknown, available evidence supports the hypothesis that DNA is the primary target (Mirkes, 1985). The critical target in terms of the antineoplastic, carcinogenic and mutagenic properties of cyclophosphamide is also DNA. Although cyclophosphamide metabolites alkylate embryonic DNA, RNA and proteins, Murthy et al. (1973) found that the extent of drug binding to embryonic DNA correlated with overall frequencies of malformations, whereas binding to RNA and protein did not. Short et al. (1972) noted that cyclophosphamide inhibits DNA and protein synthesis, but not RNA synthesis, in mouse embryos. They suggested the effects on DNA synthesis, rather than on protein synthesis, were responsible for malformations because cyclophosphamide-induced inhibition of DNA synthesis preceded the inhibition of protein synthesis. Teratogenic concentrations of phosphoramide mustard were found to induce DNA crosslinking whereas monofunctional phosphoramide mustard induced strand breaks (Mirkes et al., 1984). Despite the different primary lesions in DNA,both produced similar embryotoxic effects, suggesting that two separate DNA lesions can lead by unknown pathways to a common teratogenic outcome. It is thought that DNA lesions lead to cell death or mutations (Mirkes, 1985), which, as depicted in Fig. 1.2., lead to the final defect.

Although most studies favour DNA as the critical target of cyclophosphamide-induced teratogenicity, there is limited evidence to suggest alternative mechanisms. Kohler and Merker (1973) have published data that they suggest indicates cyclophosphamide exerts its teratogenic effects by alkylating mRNA directly. There is also some evidence that alkylation of proteins

constitutes the primary target of cyclophosphamide teratogenecity (Gurtoo et al., 1978). The induction of chromosomal aberrations is another possible mechanism. A dose dependent increase in cyclophosamide-induced chromosomal abnormalities has been demonstrated in 12-day mouse fetuses (Mayne and Legator, 1983). Similarly, Kola (1985) found an increased number of chromosomal aberrations in mouse blastocysts exposed to the teratogen.

Cyclophosphamide was chosen as a suitable teratogen for the studies in this thesis firstly, because it is a well established central nervous system teratogen; secondly, because of its relevance to human birth defects; thirdly, to compare the effects with those of vitamin A; and finally, to try and throw more light on the mechanisms of its action.

1.4. SODIUM VALPROATE AS A TERATOGEN

Valproic acid, or dipropylacetic acid, and its salt, sodium valproate, have recently gained prominence as anticonvulsants (Simon and Penry, 1975; Browne, 1980). It is known that these drugs cross the human placenta (Dickinson et al., 1979; Nau et al., 1981a), and there have been numerous case reports associating a variety of birth defects with their use during pregnancy, including craniofacial abnormalities, hydrocephalus, low birth weight and genitourinary abnormalities (Dalens et al., 1980; Clay et al., 1981; Gomez, 1981; Thomas and Buchanan, 1981; Bailey et al., 1983; DiLiberti, 1983; Hanson et al., 1984). There appears to be a substantial association between maternal valproate use and neural tube defects (Bjerkedal et al., 1982; Robert and Guibaud, 1982; Robert and Rosa, 1983; Robert et al., 1984; Lindhout, 1984) and a distinct fetal valproate syndrome has been described with characteristic facial abnormalities (DiLiberti et al., 1984). The teratogenic potential of valproate in humans has been recently reviewed (Pillans and Kola, 1985; Jager-Roman, 1986).

Valproate is known to be teratogenic in rabbits, rats and mice (Whittle, 1976; Brown et al., 1980; Nau et al., 1981b). Teratogenic doses of the drug for mice are about tenfold higher than human therapeutic doses (Schardien, 1976 : 113; Whittle, 1976). In vitro studies have demonstrated a direct embryotoxic effect on the murine embryo (Kao et al., 1981; Bruckner et al., 1983). Maternal valproate treatment during early organogenesis (days 8-10) in the CD-1 mouse, produced a dose-related increase in congenital abnormalities and a decrease in fetal weight, whereas greater embryo mortality was apparent during late organogenis (days 11-13, Kao et al., 1981). With daily administration of sodium valproate between days 7 and 15 in NMRI mice, Nau et al., (1981b) also found a dose-related increase in growth retardation, embryolethality and exencephaly. Similar findings were noted in mouse embryos in culture (Bruckner, et al., 1983). Valproate-induced murine abnormalities include exencephaly, irregular fusion of brain folds, microcephaly, stunted forebrain development, cleft palate, and rib and vertebral defects (Whittle, 1976; Sucheston et al., 1979; Brown et al., 1980; Nau et al., 1981b; Kao et al., 1981; Bruckner et al., 1983).

Histological changes in the neuroepithelium of exencephalic embryos in culture, such as alterations in the morphology of mitochondria and the occurrence of large protrusions in the lumen of the neuroepithelium, provide further evidence for a CNS teratogenic effect of valproate (Bruckner et al., 1983). As far as I can ascertain nothing is known of the teratogenic mechanisms of valproate.

From the foregoing, it would appear that sodium valproate is a central nervous system teratogen with obvious relevance to human birth defects. It was therefore chosen as a further suitable dysmorphogenic agent in this thesis to compare the effects on acetylcholinesterase and its isoenzymes with vitamin A and cyclophosphamide.

C H A P T E R 2

ANIMAL MODEL

2.1. CHOICE OF ANIMAL MODEL

The mouse is a natural choice because of its high fertility and availability in substantial numbers, as well as its known sensitivity to teratogens, including vitamin A, cyclophosphamide and sodium valproate (see Chapter 1). Also, the malformations can mimic human malformations, for example those produced by hypervitaminosis A (Shenefelt, 1972; Bernhardt and Dorsey, 1974; Stange et al., 1978).

An aspect of experimental teratology is the variability in the effects of teratogens on prenatal development. Apart from environmental factors genetic factors play an important part in this variability. To obtain homogeneity of genetic make-up inbred strains must be used, and the only inbred strains easily obtainable are strains of mice. Genetically determined responses of an inbred strain to an experimental manipulation will usually be less variable than the responses of a heterogeneous population (Kalter and Warkany, 1961).

The inbred strain I have chosen is the C3H mouse strain. The full designation is C3H/HeAfHa (ICR).

A potential disadvantage in using mice is the high sponteneous abnormality rate which in C3H mice is 3.3% (Schardien, 1976 : 23; Heinecke, 1972). However, by comparing sufficient numbers of control

and test animals this should not affect statistical analysis.

2.2. MOTIVATION FOR USING AN ANIMAL MODEL

To study the effect of teratogens on the nervous system, as for example in determining potential changes in a biochemical marker such as acetylcholinesterase, requires an animal model from which brain tissue can be obtained. Such information cannot be obtained from human studies. The problems of predicting teratogenicity of an agent in man on the basis of animal studies are well known and I will not elaborate on these. Suffice it to say that I am aware the extrapolation of these findings to man is difficult, but nevertheless might serve as a useful pointer. A further aim of this study was to help elucidate some of the mechanisms of teratogenicity, and such knowledge could be of value in understanding the mechanisms of teratogenic agents in man.

2.3. EXPERIMENTAL CONDITIONS

The mice were housed in a large artificially lit room, the lighting timed for a 12 hour cycle from 6 a.m. to 6 p.m. Air temperature was thermostatically controlled at a mean of 21^{o}C, and maximum and minimum temperatures were checked daily and recorded. Twelve to 15 air changes were provided per hour.

The female animals were housed in communal cages in groups of 15-20 per cage until 2 days prior to mating when they were placed 6 animals per cage of approximately 450 cms^{2}. The males were kept one animal per cage of approximately 450 cms^{2}. Cages were lined

with autoclaved wood shavings. All animals were fed Epol mouse cubes ad libitum, and were allowed water ad libitum. Table 2.1. shows the constituents of Epol mouse cubes. Water bottles were washed daily and water provided as necessary. Cages were cleaned two times a week and autoclaved approximately every two weeks.

Young healthy virgin female mice aged 8-12 weeks and weighing 20-23g were used for all experiments. (Puberty in the mouse starts at 4-6 weeks of age and females usually start breeding at 6-8 weeks (Williams, 1976). Mature males weighing 24.0-27.5g were used and the stock was changed approximately every 8-16 weeks according to mating performance.

2.4. MATING PROGRAM

The animals were mated monogamously once weekly. In an attempt to achieve an optimal pregnancy rate the following format was used. Between matings females were confined together in groups of 15-20 in the absence of males to induce anoestrus (Whitten, W.K., 1959). Forty-eight hours prior to mating the females were placed 5-7 animals per cage, male urine sprinkled on the bedding and the cages placed between male cages. Male urine was placed on the bedding morning and afternoon until mating. (Females tend to come into heat after approximately 48 hours of exposure to olfactory stimuli (pheromones) from the male's urine (Whitten, W.K., 1956; Whitten, W.K. et al., 1968). Females should therefore be in oestrus at the time of mating.

T A B L E 2.1.

EPOL MICE CUBES AND PELLETS

Calculated Assay	%	
Protein	18,12	
Methionine	0,35	
T.S.A.A.	0,67	
Lysine	0,89	
Tryptophan	0,22	
Arginine	1,09	
Fibre	5,92	
Calcium	0,87	
Phosphorus	0,72	
Salt	0,37	
M.E.	2484	Kcal/kg
T.D.N.	71,20	

Vitamin Premix

Vitamin A	13 793	IU/kg
Vitamin D3	1 332	IU/kg
Vitamin E	16 535	IU/kg
Vitamin B6	3,257	p.p.m.
Thiamine	2,2	p.p.m.
Riboflavine	4,274	p.p.m.
Calcium Pantothenate	19,75	p.p.m.
Niacin	23,034	p.p.m.
Vitamin B12	0,012	p.p.m.
Ethoxyquin	0,132	p.p.m.

Mineral Premix

Cobalt	0,85	p.p.m.
Copper	5,0	p.p.m.
Iron	44,0	p.p.m.
Zinc	24,0	p.p.m.
Iodine	0,61	p.p.m.
Manganese	18,5	p.p.m.

INGREDIENTS:

Energy Sources

Yellow Maize Meal, Bran (wheat), Molasses.

Plant Protein

Groundnut, Lucerne, Sunflower.

Animal Protein Fish Meal, Salt.

Timed matings were performed and were restricted to a 2h period from 5 a.m. to 7 a.m. Mice are nocturnal breeders and oestrus begins in 75% of mice 4-6h after dark (i.e. between 10 p.m. and midnight in the mice used). The mean duration of oestrus is 14h. The usual time of ovulation is 8 - 11h after the onset of heat (Hafez, 1970). An early morning mating therefore ensured a maximum chance of pregnancy.

The females were removed after 2h and examined for the presence of a copulation plug (a yellowish-white mucus plug. The vagina after copulation also appears erythematous and swollen). The following 24h period was designated as day 0 of pregnancy. The day after was therefore day 1 post conception (p.c.). Apart from copulation plugs two other parameters were used as indicators of pregnancy in mice more than 8 days p.c., namely abdominal swelling or a significant weight gain by the time of dosing. Approximately 50 females were mated per week. The pregnancy rate varied between approximately 10 and 30%.

2.5. DOSING PROGRAM

On the first day of dosing with a teratogen the female mice suspected of being pregnant were randomly and equally divided into test and control groups. Animals were weighed, numbered by means of ear-punching and kept 3 per cage thereafter. All mice used were healthy.

Vitamin A palmitate ("Arovit", 300 000 IU/ml Roche products; Isando, South Africa), was diluted with Arachis oil and administered by gastric intubation to the test animals. Controls received an equal quantity of Arachis oil (purified peanut oil).

Sodium valproate (Reckitt and Colman, United Kingdom) was dissolved in sterile water and administered by subcutaneous injection. Controls were injected with an equal quantity of sterile water.

Cyclophosphamide (Noristan, Pretoria, South Africa) was dissolved in sterile water and administered by subcutaneous injection and controls were injected with an equal amount of sterile water.

Details regarding dosage and preparation of the above agents are described under the respective experiments (see Chapters 6, 7 and 8).

2.6. SACRIFICE, REMOVAL OF FETUSES AND BRAIN REMOVAL

To evaluate teratogenic effects of drugs, termination of pregnancy before parturition is necessary, because female mice are likely to cannibalise malformed and dead offspring (Palmer, 1978; Wilson, 1973). Pregnant females were usually sacrificed 1 to 2 days prior to term, i.e. on day 18 or 19 p.c. Sacrifice on day 15 p.c. or earlier meant working with extremely small and friable brains which were technically difficult to remove. Whole heads were therefore used in these experiments.

The weight of each mouse was recorded and sacrifice was by cervical dislocation. All mice were killed within 2h in any particular experiment to obviate any difference in age. The abdominal cavity was opened and uterine horns externalised. The number of implantation sites and resorptions was noted, membranes removed and fetuses examined by gross inspection for obvious external abnormalities.

The number of live and dead fetuses was recorded and fetal abnormalities noted. All live fetuses were weighed. Generally, litters of approximately equal number were compared in the test and control groups.

Brain removal

Fetuses were decapitated to enable maximal drainage of blood. Decapitation was effected by a single swift cut at the first cervical vertebra. Brains were then removed within 30 - 60 seconds and weighed. All brains appeared macroscopically normal, even in cases with external abnormalities, except for those with exencephaly. Brains were not sectioned as the whole brain (or in some experiments the cerebellum or cerebral cortex) was solubilised and homogenised fresh for AChE assay within approximately 1h of removal.

2.7. RECORDING OF RESULTS

Maternal parameters

The weight on the day of mating, day of dosing and the day of sacrifice and the physical state of each mouse after dosing and until sacrifice were recorded. All mice were healthy at the time of sacrifice (two mice with significant abdominal swelling were noted to have lost weight and were found to be non-pregnant with intra-abdominal neoplasm). In some earlier experiments maternal weights were not recorded.

Fetal parameters

The number of implantation sites, number of resorptions, fetal weights,

brain weights and obvious external abnormality or death were recorded. Depending on the particular study, the following were investigated: brain acetylcholinesterase or its isoenzymes, cholinesterase, choline acetyltransferase, total protein content, protein patterns, and DNA damage. In the study with preimplantation embryos, viability, cell number, mitotic index and chromosome structure were examined.

2.8. BRAIN SOLUBILISATION AND HOMOGENISATION

Fetal brains (generally 20mg/ml) were solubilised and homogenised in a 2% Triton X-100/0,05M Tris-HCl buffer, pH 7,4 in earlier experiments, or an 0,5% Triton X-100/0,05 Tris-HCl buffer, pH 8,4 in later experiments. A Thomas Teflon pestle homogeniser was used at 1 425 r.p.m. for one minute per sample. Each homogenate was kept in a separate labelled glass tube at 4°C until assayed.

Note:

A. Clear homogenates were obtained. The advantages of a clear homogenate were:

(i) centrifugation was unnecessary. Total AChE activity of the homogenate was therefore determined.

(ii) good linear tracings were obtained on the spectrophotometer without interference from suspended particles.

(iii) the clear homogenate was suitable for separation of the isoenzymes of AChE by polyacrylamide gel electrophoresis.

B. Clear homogenates were obtained using fetal or neonatal mouse brains but not brains from older mice where the homogenate remained turbid,

presumably due to the presence of more fibrous connective tissues.

2.9. MINIMAL TOXIC DOSE STUDIES

The minimal toxic dose (M.T.D.) is that dose of a teratogen resulting in either significant weight reduction during the treatment period, mortality or other signs of toxicity in the parent animal (Chernoff and Kavlock, 1982). If doses used are below the M.T.D. for the mother this diminishes the chances that maternal toxicity will confound the neonatal results. Studies were therefore performed to determine whether the doses of the teratogens generally used were below the M.T.D. for the C3H mice.

Non-pregnant C3H females weighing 20-24g were removed from stock cages of ± 15 mice per cage and placed 3 per cage a few days prior to dosing to enable adjustment to these conditions. The teratogen was then administered, animals numbered by means of ear punching, and kept 3 per cage under standard conditions. They were given food and water ad libitum. The mice were observed for signs of toxicity, mortalities recorded, then reweighed ± 9 days after dosing (the usual duration between dosing and sacrifice in most experiments) and the weights compared with the pre-treatment weights. The studies with each teratogen used are presented in the respective chapters.

CHAPTER 3

ACETYLCHOLINESTERASE

3.1. INTRODUCTION

Apart from frank congenital malformations prenatal toxicity may also result in more subtle biochemical abnormalities (Snell, 1982 b). Alterations of enzyme activity, modifications of isoenzyme patterns or other enzyme changes may reflect perturbations of fundamental biochemical processes. Enzymes are one of the largest and most varied classes of specific chemical constituents involved in metabolic processes and are probably ubiquitous in all aspects of differentiation and growth. Hence they would almost certainly be interfered with by factors adversely affecting these developmental processes. Some teratogenic mechanisms may in fact involve enzyme inhibition as the primary or initiating event in abnormal embryogenesis (Wilson, 1977:70).

Detection of metabolic lesions, particularly in the absence of morphological abnormalities, would greatly extend the sensitivity of standard teratological investigations. Quantifiable deviations in the activity of organ-characteristic enzyme systems may be sensitive and reliable indicators for estimating threshold levels for deleterious effects (Andrew and Lytz, 1981). It would therefore be of interest to study enzymes as potential markers of prenatal toxicity.

There were several reasons why acetylcholinesterase (AChE) was chosen as a suitable enzyme or potential marker for studying drug injury to the fetal central nervous system (CNS), but the idea arose from the recent surge in the literature on the measurement of amniotic fluid AChE in the prenatal diagnosis of neural tube defects.

Acetylcholinesterase has been found to be elevated with such CNS abnormalities as anencephaly and spina bifida (Dale et al.,1981; Smith et al.,1979; Wald and Cuckle, 1981, and many others). However, although the finding demonstrates the presence in the fetal CNS of AChE, it appears that the abnormal presence of AChE in amniotic fluid depends on there being an open defect to enable a leak from neuronal tissue to the amniotic fluid, rather than being the result of any intrinsic abnormality in the CNS. Thus, although the recent work on amniotic fluid sowed the seed, it cannot be considered a valid reason for choosing AChE.

The first reason why AChE was chosen was because the enzyme is an integral component of CNS function, and could therefore serve as a useful biochemical marker for toxic or teratogenic effects on neuronal tissues. Components essential for neurotransmission, which includes enzymes, serve as specific biochemical markers for neuronal systems (Johnston and Coyle, 1981). The cholinergic system provides significant innervation to most regions of the brain and AChE is needed for the rapid hydrolysis of the acetylcholine released during neurotransmission. AChE activity is thought to be directly related to the amount of acetylcholine present (Burkhalter et al.,1957), or to be a measure of acetylcholine

metabolism in the brain (Bennett et al., 1958a; Bennett et al., 1958b). It has been postulated that central neurotransmitter disturbances may account for the teratogenic effects of drugs acting on the developing brain (Mirmiran, 1984). Secondly, AChE is an index of fetal brain development. A steady increase in the enzyme parallels CNS development in the fetus and is thought to be related to maturation of nervous pathways (Bus and Gibson, 1974; Pryor, 1968; Metzler and Humm, 1951). Any perturbation of normal brain development could therefore potentially be accompanied by an alteration in AChE. For example, Eckert et al., (1976) noted changes in rat brain AChE activity resulting from undernutrition imposed during gestation. Thirdly, the well known teratogenic agent, X-irradiation, has been shown to produce changes in rat brain AChE. (Maletta and Timiras, 1966; Maletta et al., 1967; Altman and Anderson, 1969; Adlard and Dobbing, 1972). Finally, if one of the teratogenic mechanisms causing abnormal embryogenesis is enzyme inhibition (Wilson, 1977 : 70) or an abnormality of enzyme function, this should be evident.

3.2. CHOLINESTERASES : DEFINITION AND NOMENCLATURE

A composite definition which incorporates their main characteristics is that cholinesterases are hydrolases which, under optimal conditions, catalyse the hydrolysis of choline esters at a higher rate than that of other esters and which are inhibited by low concentrations (10^{-5}M or less) of inhibitors such as physostigmine and organophosphorus compounds. They constitute a family of enzymes which fall broadly into two types: those which preferentially hydrolyse acetyl esters such as acetylcholine (ACh) and those with a preference for other types of esters such as butyrylcholine (BuCh).

The naming of cholinesterases represents a long standing controversy. True cholinesterase has been given various names. In 1964 the Enzyme Commission proposed acetylcholinehydrolase (EC 3.1.1.7) as the systematic name with acetylcholinesterase as the trivial name. This latter name, or its abbreviation, AChE is the one used in this thesis. The term pseudocholinesterase has been widely used for the non-specific types of enzyme. The term "pseudo" however, implies "false" and this makes the term rather unacceptable. Perhaps a better term is butyrylcholinesterase. The Enzyme Commission (1964) recommended acylcholine acylhydrolase (EC 3.1.1.8) as the systematic name and simply cholinesterase as the trivial name. I have chosen to use the term cholinesterase (ChE) for the non-specific enzyme, in contradistinction to acetylcholinesterase for the specific enzyme.

3.3. THE VARIABILITY OF ACETYLCHOLINESTERASE ACTIVITY

A knowledge of various biological factors which may influence AChE activity is important as these are potential variables which must be accounted for and standardised when studying the effects of teratogens on the enzymes' activity. The activity of AChE is affected by physical factors which as temperature, pH and substrate concentration. Activity increases with increasing temperature, the optimum temperature for AChE activity for mammalian tissues being 37-40^{o}C (Augustinsson, 1948). With acetylthiocholine as substrate the activity of AChE increases with pH up to 7, but thereafter is unaffected by any further rise in pH and remains maximal (Bergman et al., 1958). The activity of AChE is

affected by substrate concentration, inhibition occurring when substrate is present in excess (Ellman, 1961; Garry and Routh, 1965).

A number of biological factors bear relevance to enzyme activity. Age is important since a steady increase in brain AChE activity has been noted during development (Metzler and Humm, 1951; Pryor, 1968; Bus and Gibson, 1974; Sung and Ruff, 1983). Strain differences in AChE activity have been noted in both the rat and the mouse (Pryor et al.,1966; Pryor 1968; Ebel et al.,1973; Waller et al., 1983). The possibility that seasonal changes in enzyme activity occur (Silver, 1974), and that embryotoxicity may be subject to seasonal modifications (Sauerbier, 1981) may be of relevance to any long term study. There do not appear to be significant sex differences in brain AChE activity (Woolley, 1963; Pryor, 1968). Maternal undernutrition results in changes in brain AChE in the offspring (Sereni et al.,1966; Adlard and Dobbing, 1971a,b; Eckhert et al.,1976).

Because of the influence of the above parameters the variability of AChE activity was accounted for in this study as follows:

(i) A control group under identical conditions was included with each experiment and comparisons of enzyme activity made only with the control group in that particular experiment.

(ii) Experiments were performed at a standard temperature and pH, using an optimal substrate concentration as determined in section 3.4.8.

(iii) Timed matings were performed so that all fetuses were the same age in any particular experiment.

(iv) Only one strain of mouse, C3H, was used.

(v) By comparing treated fetuses with controls each time an experiment was performed to some extent obviated the possibility of a confounding seasonal effect.

(vi) All animals received a balanced diet and were given food and water ad libitum (see Chapter 2).

3.4. QUANTITATIVE MEASUREMENTS OF ACETYLCHOLINESTERASE

3.4.1. Introduction

There are numerous methods for recording AChE activity, but the colorimetric method based on the Ellman reaction (Ellman, 1959; Ellman et al.,1961; Garry and Routh, 1965; Dietz et al.,1973) was chosen because it is extremely sensitive and is rapid and simple. The method has been used to study AChE activity in rat and mouse brain homogenates (Ellman, 1961; Maletta and Timiras, 1966; Pryor, 1968; Valcana and Timiras, 1974; and others).

Acetylcholinesterase hydrolyses acetylthiocholine to yield free thiocholine. The enzyme activity is measured by following the increase of yellow colour produced from thiocholine when it reacts with dithiobisnitrobenzoate ion. It is based on coupling of these reactions:

$$\text{acetylthiocholine} \xrightarrow{\text{(AChE)}} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + \text{dithiobisnitrobenzoate} \longrightarrow \text{yellow colour}$$

The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolyzed. This is accomplished by the continuous reaction of this thiol with DTNB (5:5-dithiobis-2-nitrobenzoate ion) to produce the yellow anion of 5-thio-2-nitro-benzoic acid.

$$H_2O + (CH_3)_3\overset{+}{N}CH_2CH_2SCOCH_3 \xrightarrow{(AChE)} (CH_3)_3\overset{+}{N}CH_2CH_2S^-$$

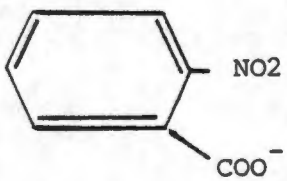
acetylthiocholine — thiocholine + $\overset{+}{C}H_3COO^-$ + $2H^+$

acetate

$$(CH_3)_3\overset{+}{N}CH_2CH_2S^- + RSSR \longrightarrow (CH_3)_3\overset{+}{N}CH_2CH_2SSR + RS^-$$

thiocholine — DTNB — 5-thio-2-nitro-benzoate

R =



The reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme, and in the concentrations used does not inhibit the enzymic hydrolysis (Ellman, 1961; Garry and Routh, 1965).

The rate of colour production is measured at 410nm (the absorption maximum of the yellow nitro-benzoate - see section 3.4.7 . DTNB absorbs maximally at 320nm).

3.4.2. Materials and methods

Fetal mouse brains were solubilised and homogenised as described

in section 2.8. A 0,4ml aliquot of the homogenate was added to each of two cuvettes, containing 3,0ml of phosphate buffer (pH 8,0; 0,1M) and 100µl of 10mM dithiobisnitrobenzoic acid (DTNB). All aliquots of 0,4ml and smaller were measured by means of micropipettes. (The one cuvette serves as the test sample and the other as a blank. The blank corrects for any spontaneous degradation of DTNB, for the release of any thiol material from fetal tissue, and for the absorbance of other materials in the homogenate). The two cuvettes, prewarmed in a water bath to 37°C, were placed in the spectrophotometer, (a Cary Varian 219 spectrophotometer was used) one as the test sample and the other as the blank. The reaction was started by adding 50µl of 0,075M acetylthiocholine iodide (measured in a Hamilton syringe) to the test cuvette. The contents were mixed thoroughly by up and down strokes with a specially designed mixer (made from glass capillary tubing with the end flattened at right angles to the shaft). The absorbance was recorded at 410nm against the blank. (Linear tracings were obtained with fetal mouse brain homogenate).

Approximately equal numbers of test and control homogenates were assayed in each experiment, alternating 5 test with 5 control samples until all assays were completed. This ensured that an equal number of test and control samples were subjected to the same time delay prior to assay. The maximum delay between brain removal and assay was approximately 4 h. All samples were stored in the fridge at 4°C until assayed.

3.4.3. Calculation of AChE activity

Rates are calculated as follows:

Change in absorbance/minute (ΔA) is calculated from the slope of the spectrophotometric tracing.

The following explanation of the calculation is taken from Silver (Silver, 1974 : 63), and is based on Ellman's work (Ellman et al., 1961). Results of the rate of enzymic hydrolysis are expressed in the more common form of nmol/min/mg.

The extinction coefficient for a molar solution (1 mole/litre) of coloured anion is $1{,}36 \times 10^{4}$ (Ellman, 1959), therefore the extinction coefficient for 1 nmol in a cuvette of volume Vml =

$$\frac{1{,}36 \times 10^{-2}}{V}$$

The rate of hydrolysis of substrate is therefore

$$\frac{\Delta A \times V}{1{,}36 \times 10^{-2}} \quad \text{nmol/min}$$

If the concentration of the enzyme preparation is Cmg/ml and the volume used is Yml the rate of hydrolysis in nmol/min/mg is

$$\frac{\Delta A \times V}{1{,}36 \times 10^{-2}} \times \frac{1}{C \times Y}$$

Calculation of enzyme activity can also be calculated in units per litre (Bonham et al.,1981).

(The units are arbitrary and are calculated per unit volume (e.g. litre) if a weight of sample cannot be obtained, e.g. using serum).

Enzyme activity in units/l

$$= \frac{\text{Total vol, V (ml)}}{\text{Sample vol, V}(\mu l)} \times \frac{10^9}{Em} \times \Delta A$$

(Em = $1{,}36 \times 10^4$ - the extinction coefficient of the anion of 5-thio-2-nitro-benzoic acid).

If results are to be expressed in units:

The standard unit (U) is defined as follows:

One U of an enzyme is that amount which will catalyse the transformation of 1 µmol of the substrate per minute under standard conditions. e.g. 1 unit of AChE will hydrolyse 1 µmol of acetylcholine to choline and acetate per minute at pH 8,0 at 37°C.

Note:

Ellman et al, (1961) noted that the rate of hydrolysis given by the above method using acetylthiocholine as opposed to acetylcholine as substrate can be taken as a good indication of the rate of hydrolysis of AChE. However, as the authors themselves showed, the kinetic constants obtained with the two substrates are not identical and this must be recognised in any comparison with results from other methods.

3.4.4. Accuracy, sensitivity and reproducibility of spectrophotometric measurement of acetylcholinesterase

To determine the accuracy, sensitivity and reproducibility of the spectrophotometric assay of AChE, two experiments were performed.

In the first experiment the activity of pure AChE (Sigma, 0,72 U/mg)

was checked.

Method:

1 mg pure AChE was dissolved in 5ml distilled water (concentration 0,2mg/ml). 400µl of this was added to 3,0ml phosphate buffer (0,1M, pH8,0) and 100µl of DTNB. The reaction was started by adding 50µl AThCh (75mM) and the change in absorbance was measured at 37°C. The experiment was repeated six times. Enzyme activity was calculated in nmol/min and converted to enzyme units (U).

Result:

The mean activity of AChE was 0,70U/mg (± 0,01). The result is acceptably accurate, being very similar to the stated activity of the Sigma AChE, 0,72U/mg.(The slightly lower activity is probably explained by some decrease in activity with storage).

In the second experiment a range of dilutions of pure AChE (Sigma, 0,72U/mg) was assayed to determine the degree of sensitivity of the spectrophotometric assay.

Method

2,13mg pure AChE (Sigma) was dissolved in 4,0ml distilled water and then diluted as follows: 1/2; 1/4; 1/10; 1/20; 1/100; 1/200. The Ellman assay was performed as described above and was repeated three times for each dilution. The rate of spontaneous hydrolysis of AThCh (0,007 ΔA/min, see section 3.4.6.),was subtracted from the rate of reaction for each dilution to give the corrected ΔA/min. At the lower dilutions (i.e. 1/20; 1/100; 1/200) the sensitivity range

of the spectrophotometer was increased i.e. from 1,0 to 0,5 to 0,1.

Results are shown in Table 3.1. A good correlation (r = 0,9995) between recovered and initial dilutions at least to a dilution of 1/20 was apparent.

Conclusion

The sensitivity of the assay was very satisfactory to a dilution of at least 1/20 and was acceptable for the studies in this thesis. Reproducibility was acceptable at all dilutions tested.

3.4.5. Determination of acetylcholinesterase activity in C3H fetal mouse brains and comparison of activity between individuals of the same age

In order to study the effects of teratogens on AChE in fetal mouse brain there should ideally be (i) substantial amounts of enzyme present and (ii) limited variation in enzyme activity between individuals of the same age. To investigate these parameters AChE activity was determined in 20 fetuses.

Method

The brains from twenty 19-day C3H mouse fetuses from 4 litters were solubilised and homogenised as described in section 2.8. Acetylcholinesterase activity was determined as described in section 3.4.2.

Results are shown in Table 3.3. Fetal mouse brain AChE activity is compared with pure bovine AChE activity in Table 3.2.

T A B L E 3.1.

TABLE SHOWING ABSORBANCE (ΔA/MIN) AT INCREASING DILUTIONS OF PURE ACETYLCHOLINESTERASE AND THE RESULTING RECOVERED DILUTIONS AND COEFFICIENTS OF VARIATION

No.	Corrected* ΔA/min	Initial Dilution	Recovered Dilution	Coefficient of variation (%) (= SD / Recovered dilution)
1	0,525 0,525 0,527	1	1,006 1,006 1,01	0,3
2	0,247 0,254 0,265	0,5	0,477 0,49 0,511	0,31
3	0,1735 0,126 0,120	0,25	0,223 0,246 0,235	0,55
4	0,052 0,0485 0,049	0,1	0,105 0,0985 0,0994	1,4
5	0,0233 0,0222 0,0226	0,05	0,0505 0,0484 0,0491	3,0
6	0,008 0,0056 0,0049	0,01	0,0213 0,0168 0,0154	9,5
7	0,0043 0,0013 0,0044	0,005	0,0143 0,0086 0,0145	14,8

Linear Regression: Correlation coefficient r = 0,9995
Slope (SD) = 0,525 (0,004)
Intercept (SD) = -0,003 (0,002)

* Recorded ΔA - ΔA of spontaneous hydrolysis.

T A B L E 3.2.

COMPARISON OF FETAL MOUSE BRAIN ACETYLCHOLINESTERASE ACTIVITY WITH PURE BOVINE RED CELL ACETYLCHOLINESTERASE ACTIVITY

Source of AChE	AChE activity		
	A/min	U/l	nmol/min/mg
Pure Bovine AChE 0,2mg/ml (0,72 Units/mg	0,212	138,32	682,0
Brain 40mg/ml	0,218	142,26	3,55
Brain 20mg/ml	0,104	67,87	3,37

Comment and conclusion

The brain AChE results of 19-day C3H mouse fetuses (mean 3.37 nmol/min/mg) confirmed substantial AChE activity. Limited inter-individual variation in brain enzyme activity was apparent (S.D.=0,23, see Table 3.3.). The high value for bovine red cells was explicable on the basis that pure enzyme was used as opposed to AChE in brain which was considerably attenuated by brain tissue.

The substantial enzyme activity and limited inter-individual variation suggested that AChE was suitable for the quantitative studies in this thesis.

3.4.6. Non-enzymic hydrolysis of the substrate acetylthiocholine iodide

Some non-enzymic or spontaneous hydrolysis of the substrate acetylthiocholine iodide (AThCH) is known to occur (Ellman et al., 1961; Garry and Routh, 1965).

TABLE 3. 3.

ACETYLCHOLINESTERASE ACTIVITIES CALCULATED FOR TWENTY 19-DAY FETUSES FROM FOUR DIFFERENT LITTERS

Litter No.	Fetus No.	ΔA/min	AChE activity nmol/min/mg
1	1	0,107	3,49
	2	0,108	3,52
	3	0,1075	3,51
	4	0,104	3,39
	5	0,1115	3,64
	6	0,1105	3,60
2	7	0,1005	3,28
	8	0,101	3,29
	9	0,101	3,29
	10	0,094	3,07
	11	0,100	3,26
	12	0,101	3,29
	13	0,106	3,46
3	14	0,108	3,52
	15	0,103	3,36
	16	0,101	3,29
	17	0,101	3,29
4	18	0,097	3,16
	19	0,1095	3,57
	20	0,100	3,26
Mean S.D.		0,104 0,005	3,37 * 0,23

* This AChE activity is equivalent to 67,87 U/l.

The extent of this hydrolysis was determined under the conditions of this study and the contribution to total hydrolysis calculated.

Method

Non-enzymic hydrolysis was demonstrated by reading the change in absorbance, ΔA, of a test cuvette containing 3.4ml phosphate buffer, pH 8.0, 50µl AThCh (75mM; stock conc.) and 100µl DTNB at 37°C against a blank containing 3.45ml phosphate buffer and 100µl DTNB at 410nm. The assay was performed on 6 samples.

Results

Sample No.	ΔA/min	
1	0,001	
2	0,0035	
3	0,002	
4	0,0024	
5	0,0015	
6	0,0019	
Mean ΔA =	0,00205	(S.D. 0,00085)

The following are six representative ΔA results for 18 day fetal mouse brains using 400µl brain homogenate (20mg/ml), 3ml phosphate buffer, 50µl AThCh and 100µl DTNB.

Brain Sample No.	ΔA/min	
1	0,101	
2	0,106	
3	0,108	
4	0,103	
5	0,101	
6	0,101	
Mean ΔA =	0,103	(S.D. 0,003)

Spontaneous hydrolysis of the substrate acetylthiocholine accounts on average for

$$\frac{0,00205}{0,103} \times 100$$

= 1,97% of total change in absorbance.

Comment

Spontaneous hydrolysis is the same for each sample since 50µl of substrate are used in every case. It can be seen that this non-enzymic hydrolysis contributes a very small amount to the total hydrolysis (enzymic plus non-enzymic) of a sample. AChE results were therefore not corrected for this component which was accepted as representing an insignificant constant. In any case it would not affect statistical tests based on comparison of mean values.

3.4.7. Determination of the absorption maximum of the 5-thio-2-nitro-benzoate ion

The absorption maximum of the 5-thio-2-nitrobenzoate ion is variably quoted as 410 or 412nm by different workers (Dietz et al., 1973; Ellman et al., 1961; Silver, 1974 : 63; Garry and Routh, 1965).

Method

Using the Varian spectrophotometer the visible light absorption was read in a cuvette containing 3ml phosphate buffer, pH 8,0; 0,4ml 18 day C3H fetal mouse brain homogenate (20mg/ml); 50µl acetylthiocholine iodide and 100µl DTNB at 37°C. The experiment was performed 6 times.

The AChE in the brain hydrolyses acetylthiocholine to yield free thiol which reacts with DTNB to yield the 5-thio-2-nitrobenzoate ion as described in section 3.4.1.

Results

With each of the six experiments the absorption maximum was 410nm, as shown in the spectrophotometric tracing, Fig. 3.1. This wavelength was therefore chosen for all spectrophotometric experiments using DTNB.

3.4.8. Determination of the optimal substrate concentration of acetylthiocholine iodide (AThCh)

An important characteristic of AChE is that it shows increasing activity with increasing substrate concentration at lower substrate concentrations, but demonstrates enzyme inhibition when substrate is present in excess (Ellman, 1961; Garry and Routh, 1965; Silver, 1974 : 10). It was therefore necessary to determine the optimal substrate concentration of AThCh for C3H fetal mouse brain AChE.

Materials and Methods

1. (i) Pooled fetal mouse brains were solubilised and homogenised in 0,05M Tris-HCl, 0,5% Triton X-100 (pH 8,4)to give a final concentration of 20mg/ml brain homogenate.

(ii) AChE activity (i.e. hydrolysis of substrate) was assayed using the Ellman technique as described in section 3.4.2., with varying substrate concentrations. Rate of hydrolysis was expressed as a change in absorbance (ΔA) per minute. The actual rate of hydrolysis for each substrate concentration was calculated by subtracting the rate of spontaneous hydrolysis of substrate from the measured rate of hydrolysis.

(iii) Spontaneous (non-enzymic) hydrolysis of the substrate was measured in the absence of brain homogenate.

(iv) Results are shown graphically in Fig. 3.2.

Fig. 3.1. DETERMINATION OF THE ABSORPTION MAXIMUM OF THE 5-DITHIO-2-NITRO-BENZOATE ION

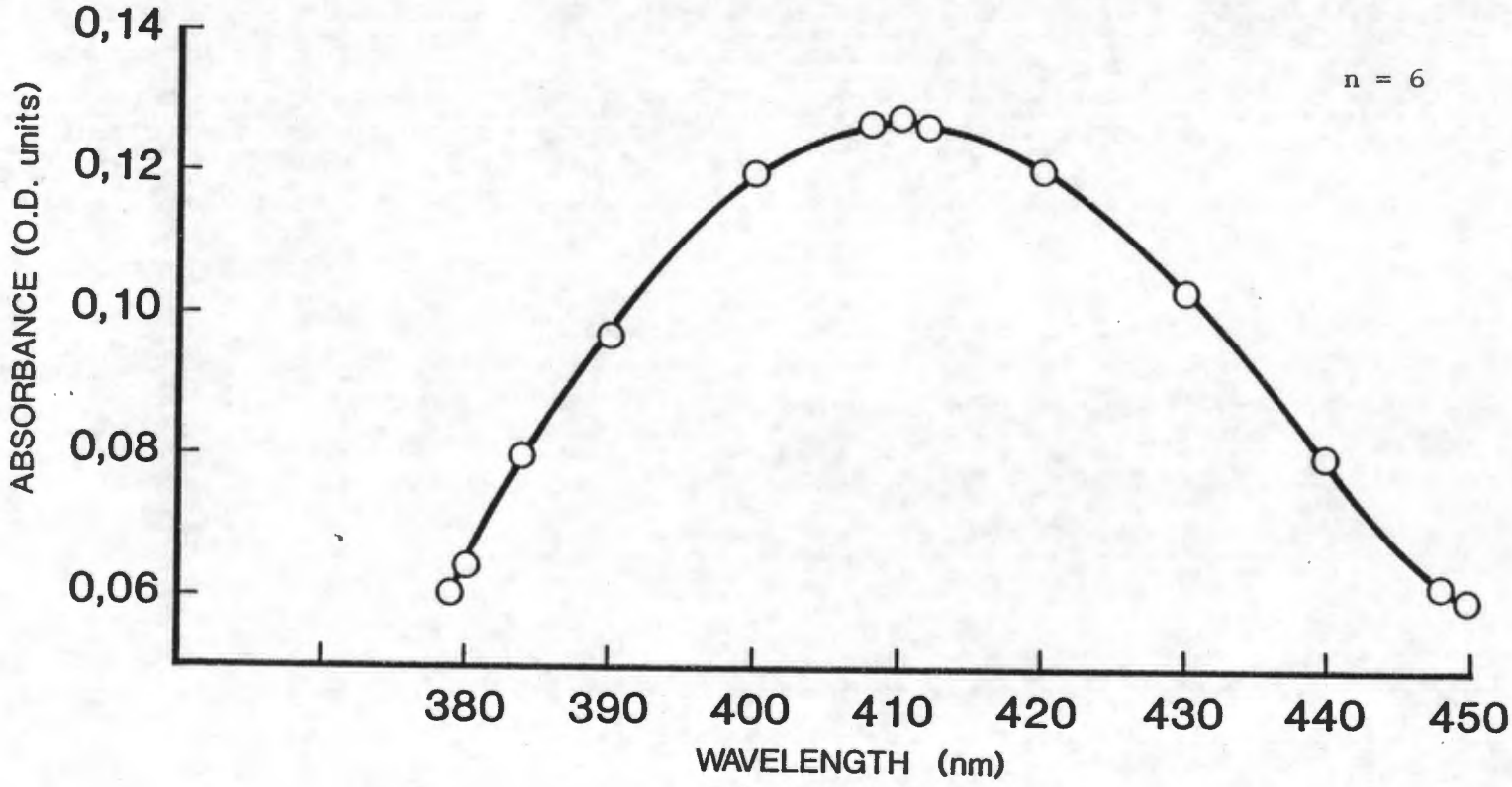
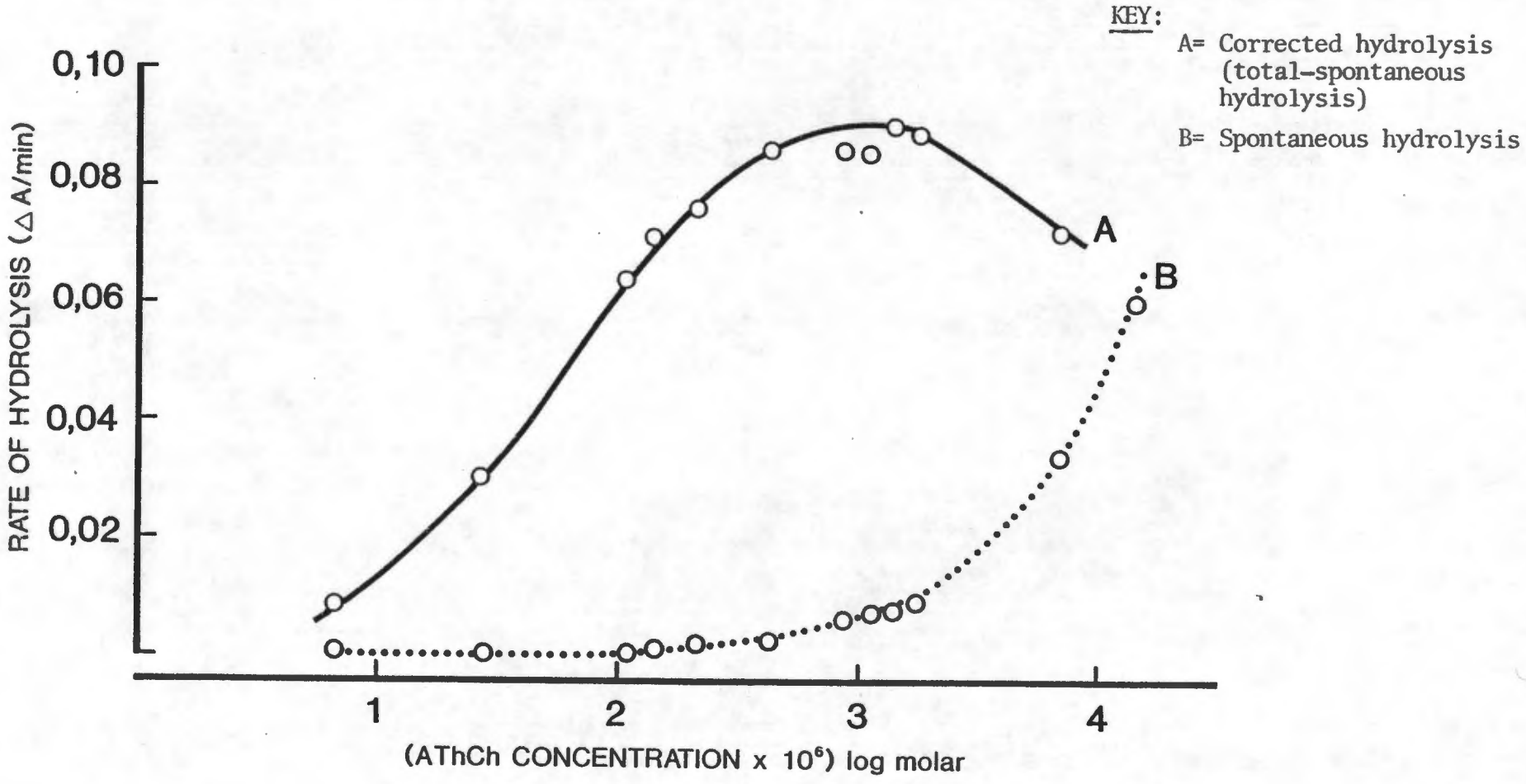


Fig. 3.2. DETERMINATION OF THE OPTIMUM SUBSTRATE CONCENTRATION FOR FETAL MOUSE BRAIN AChE (Brain homogenate Conc. = 20mg/ml)



2. The above experiment was repeated to determine the rate of hydrolysis at varying substrate concentrations for three different brain homogenate concentrations (10, 20, 40mg/ml). Results are given in Table 3.4. and shown graphically in Fig. 3.3.

Results

(i) As demonstrated in Fig. 3.2., enzyme activity increases with increasing substrate concentrations at lower AThCh concentrations, but enzyme inhibition occurs at high substrate concentrations.

(ii) Using this method the optimum stock concentration of substrate was found to be 73mM.

(iii) The rate of spontaneous hydrolysis increases with increasing substrate concentration. This increase becomes exponential at high substrate concentrations (i.e. AThCh > 100mM).

(iv) As demonstrated in Figs. 3.2. and 3.3., the optimal substrate concentration is approximately the same for the three brain homogenate concentrations. As expected, the maximum rate of hydrolysis (ΔA/min) is approximately double for each two fold increase in brain homogenate concentration (Fig. 3.3.)

Similarly, the Km (substrate concentration at which the rate of hydrolysis is half the maximum rate) is similar for the three homogenate concentrations.

3.4.9. The effect of storage on acetylcholinesterase activity

The effect of freezing and storage at -20°C for 48 hours

Acetylcholinesterase activity of twenty C3H 18 day fetal brain homogenates

TABLE 3.4.

THE EFFECT OF SUBSTRATE CONCENTRATION ON AChE ACTIVITY IN 18-DAY FETAL MOUSE BRAIN

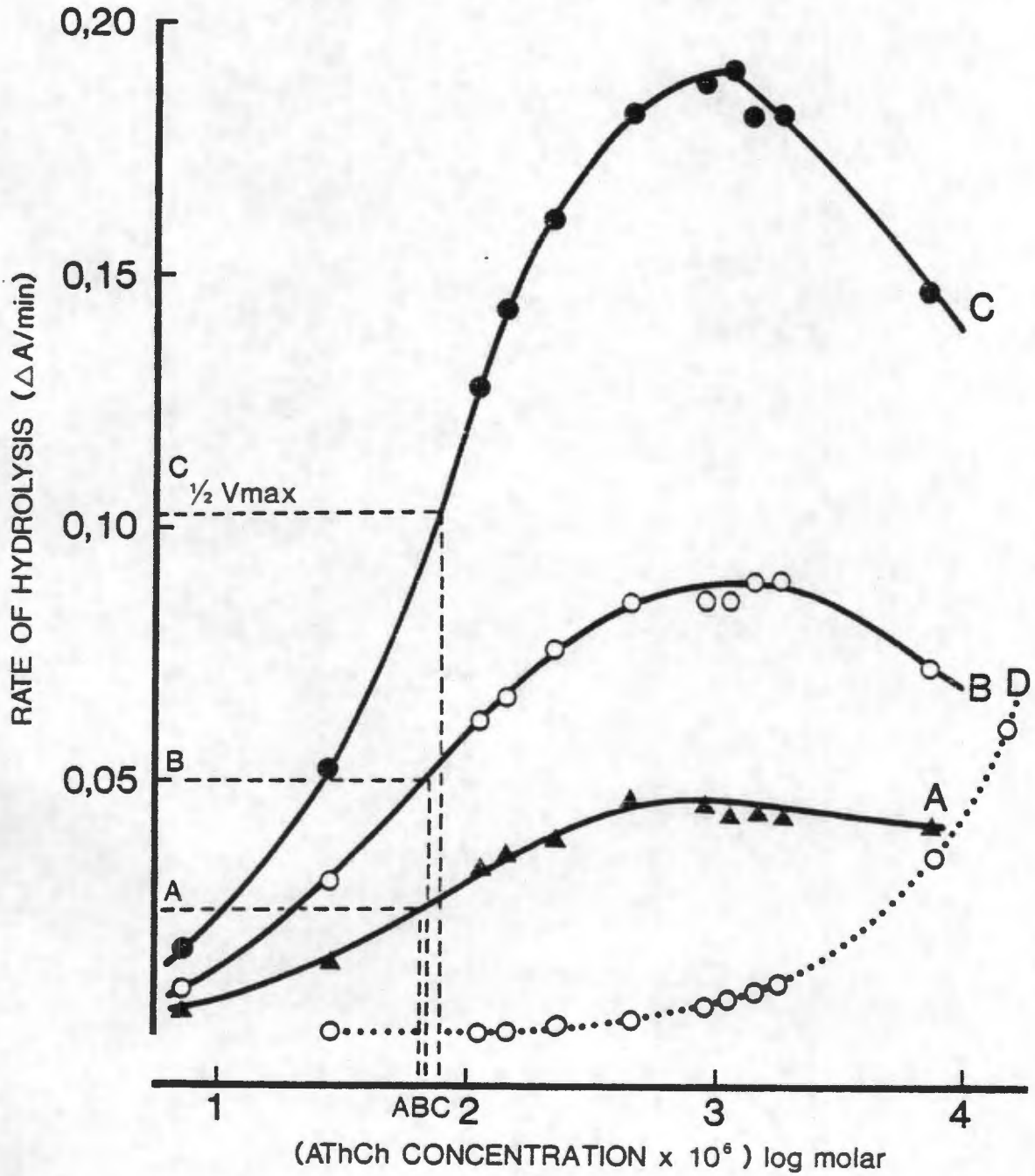
[AThCh] M	Log 10 ([AThCh] x10^6)	Spontaneous Hydrolysis	Brain 10mg/ml	Brain 20mg/ml	Brain 40mg/ml
		ΔA/min	ΔA/min	ΔA/min	ΔA/min
$6,89x10^{-6}$	0,84	0,001	0,004	0,0085	0,016
$2,76x10^{-5}$	1,44	0,000	0,016	0,03	0,0515
$1,10x10^{-4}$	2,04	0,0005	0,032	0,062	0,1275
$1,4x10^{-4}$	2,15	0,001	0,035	0,067	0,142
$2,19x10^{-4}$	2,34	0,002	0,038	0,076	0,161
$4,41x10^{-4}$	2,64	0,003	0,044	0,086	0,182
$8,82x10^{-4}$	2,94	0,006	0,044	0,086	0,1875
$1,11x10^{-3}$	3,04	0,0075	0,043	0,0855	0,189
$1,41x10^{-3}$	3,15	0,008	0,043	0,09	0,1815
$1,76x10^{-3}$	3,24	0,009	0,042	0,0885	0,182
$7,06x10^{-3}$	3,85	0,034	0,039	0,072	0,147
$1,41x10^{-2}$	4,15	0,061			

Fig. 3.3.

THE EFFECT OF SUBSTRATE CONCENTRATION ON 18-DAY FETAL MOUSE BRAIN AChE USING 3 DIFFERENT BRAIN HOMOGENATE CONCENTRATIONS

KEY: A= 10mg/ml brain homogenate
B= 20mg/ml brain homogenate
C= 40mg/ml brain homogenate
D= Spontaneous hydrolysis

(The rate of hydrolysis for curves A, B and C was corrected for spontaneous hydrolysis)



was first determined as described in section 3.4.2. on the fresh samples and repeated after storage. Results are given in Table 3.5.

(Note: Brains were homogenised in 0,5% Triton X-100/0,05M Tris-glycine, pH 8,6 which accounts for the high enzyme activity (see section 3.4.11.)

Comment

As can be seen in Table 3.5. a 20,99% (median estimate) decrease in activity occurred after 48h at -20°C compared with that in fresh samples. These findings are in agreement with other workers. Cryer and Bartley (1974) noted diminished enzyme activity of rat tissue due to inactivation with storage at -20°C to -190°C for any length of time. All assays have therefore been performed on fresh samples. (Of interest is that this decrease in activity contrasts with human amniotic fluid which is thought not to be associated with decline in AChE activity when stored at -20°C for up to years. Presumably the action of proteases, lysozymes, etc. released by cell disruption during homogenisation of brain accounts for the loss of activity. These substances would not be anticipated to be present in significant concentrations in amniotic fluid.)

The effect of storage at 4°C for up to 4h on acetylcholinesterase activity in fetal mouse brain homogenates

With a particular group of fetuses all were sacrificed within a short period of time (± 1h) so as to minimise any discrepancy in age. The brains were removed immediately, solubilised and homogenised, and

T A B L E 3.5.

THE EFFECT OF STORAGE FOR 48 HOURS AT -20°C ON AChE ACTIVITY IN BRAIN HOMOGENATES

Sample No.	AChE activity (nmol/min/mg)		% decrease in activity
	Initial	After storage	
C1	3,908	2,390	38,84
C2	5,745	4,913	14,48
C3	5,85	4,761	18,62
C4	6,661	4,296	35,51
C5	5,934	4,655	21,55
C6	6,381	5,077	20,44
C7	6,498	4,142	36,26
C8	7,134	6,092	14,61
C9	6,094	4,800	21,24
C10	6,008	4,486	25,33
T1	6,153	5,606	8,89
T2	6,464	4,98	22,96
T3	6,078	5,562	8,49
T4	6,414	4,247	32,73
T5	6,480	4,860	25,00
T6	6,299	5,508	12,56
T7	6,057	5,184	14,41
T8	5,655	4,438	21,52
T9	6,04	4,778	20,89
T10	5,694	4,807	15,58
Median	6,119	4,828	20,99
95% Confidence limits	5,929 6,317	4,548 5,135	16,95 25,68

the homogenates stored separately in numbered glass tubes at 4°C in the refrigerator.

When large numbers of assays were to be performed, the delay from time of sacrifice to time of enzyme assay was as long as four hours.

To observe whether any change in enzyme activity occured during a 4h period of storage, assays were performed on fresh brain homogenates, and repeated after 4h storage at 4°C. AChE activity was determined by the Ellman method as described in section 3.4.2.

Results are presented in Table 3.6.

T A B L E 3.6.

TABLE SHOWING COMPARISON OF CHANGE IN ABSORBANCE /MIN (Δ A) OF FRESH HOMOGENATE AND THE SAME HOMOGENATE FOUR HOURS LATER

Assay No.	Δ A of Fresh Homogenate	ΔA of same Homogenate after four hours
1	0,046	0,047
2	0,047	0,046
3	0,046	0,046
4	0,046	0,048
5	0,047	0,046
6	0,047	0,047
Mean	0,0465 (± 0,0016)	0,0467 (± 0,0019)

Comment

It is apparent that storage of up to 4h was not associated with any significant change in enzyme activity.

3.4.10. The effect of water and various buffers on acetylcholinesterase activity

It became apparent during preliminary experiments that AChE activity was not the same in different buffers. To demonstrate this 1mg pure bovine AChE (Sigma) was dissolved in 1ml distilled water. 200µl of this solution was added to 800µl of each of the following buffers to give a final concentration of 0,2mg/ml pure AChE:

Water			
Tris-HCl	,	0,01M,	pH7,4
Tris-HCl	,	0,01M,	pH8,6
Tris-HCl	,	0,05M,	pH8,6
Tris-glycine,		0,05M,	pH8,6

Each sample was assayed separately as follows: 0,4ml of enzyme/buffer solution was placed in a cuvette containing 3ml phosphate buffer, pH8,0, 100µl DTNB and 50µl acetylthiocholine iodide.

The change in absorbance, ΔA, was determined spectrophotometrically and enzyme activity calculated in U/l.

Results are shown in Table 3.7.

T A B L E 3.7.

CHANGE IN ABSORBANCE, ΔA, AND ACETYLCHOLINESTERASE ACTIVITY IN U/L WITH DIFFERENT BUFFERS

Buffer	Δ A	Activity in U/l
Water	0,281	183,37
Tris-HCl ,0,01M, pH 7,4	0,268	174,88
Tris-HCl , 0,01M, pH 8,6	0,265	172,93
Tris-HCl , 0,05M, pH 8,6	0,241	157,27
Tris-glycine, 0,05M, pH 8,6	0,353	230,36

Comment

As expected, some difference in enzyme activity is apparent with variation in pH or molarity. However, a striking increase in activity is noted with Tris-glycine which is not readily explicable.

The effect of different buffers on fetal mouse brain AChE was determined in the next experiment.

3.4.11. Determination of the effect of Triton X-100 and different buffers on fetal mouse brain AChE activity

Introduction

Most neuronal AChE is believed to exist in membrane bound form although a small percentage is soluble (Toschi, 1959; Chubb and Smith, 1975). Solubilisation is therefore necessary to release the enzyme. The most widely adopted solubilisation procedure is that using the non-ionic detergent Triton X-100 (Ho and Ellman, 1969; Crone, 1971; Chubb and Smith, 1975; Srinivasan et al.,1972; Rieger and Vigny, 1976; Vijayan and Brownson, 1974). Cotman et al., (1971) found that

over 75% of AChE was solubilised at pH8,0 while Srinivasan et al (1976) noted that a final concentration of Triton of 1% was capable of complete or nearly complete extraction of AChE.

Method

To determine the effect of Triton X-100 and different buffers on fetal mouse brain AChE activity, fetal mouse brains were weighed and homogenised separately in each of the following:

0,05M Tris-HCl (pH8,6)/0,5% Triton X-100; 0,05M Tris-HCl, pH8,6; H_2O; 0,05M Tris-glycine (pH8,6)/0,5% Triton X-100; 0,05M Tris-glycine, pH8,6.

0,4ml brain homogenate was placed in a cuvette containing: 3ml phosphate buffer, pH8,0, 100µl DTNB and 50µl acetylthiocholine iodide. The change in absorbance, Δ A, of each brain sample was determined spectrophotometrically, and the AChE calculated in nmol/min/mg. Results are given in Table 3.8.

T A B L E 3.8.

ACETYLCHOLINESTERASE ACTIVITY IN NMOL/MIN/MG FOR BRAINS HOMOGENISED IN VARIOUS MEDIA

Sample No.	Tris-HCl/ Triton X	Tris/HCl	H_2O	Tris-glycine/ Triton X	Tris-glycine
1	2,87	2,35	2,22	6,09	5,08
2	3,31	2,42	2,32	6,01	5,45
3	3,06	2,52	2,69	5,93	6,87
4	3,36	2,41	2,13	6,50	4,69
5	3,65	2,00	2,46	6,38	5,62
Mean	3,25	2,34	2,364	6,182	5,542
S.D.	0,298	0,201	0,22	0,246	0,824

Results and discussion

(i) Similar AChE activities were obtained using water and Tris-HCl without Triton, which were approximately one third lower than using Tris-/HCl with Triton. The higher enzyme activity with Triton X-100 can be explained by the solubilising effect of the detergent with release of membrane bound enzyme. The increase in activity of the enzyme in homogenates utilising Triton X-100 has been used to attest to the efficacy of solubilisation with the detergent (Crone, 1971; Harwood and Hawthorne (1969). Jackson and Aprison (1966) postulated that the non-ionic detergent enhances AChE activity by virtue of its surface active properties. Kazennov et al.,(1979) support this idea stating that the detergent causes lysis of the structural elements of nervous tissue which are not destroyed by homogenisation. These authors also suggested two other mechanisms of activation, namely the exposure of previously masked active sites of the enzyme, and the destruction of vesicular membrane structures containing AChE located on the inner surface of the vesicles.

(ii) High enzyme activities using Tris-glycine, with or without Triton are apparent, as noted with pure AChE in the previous section, and are presumably due to an enhancing effect of glycine either on AChE activity, or on one of the steps in the Ellman reaction. Although this is of interest it is a side issue and I have not pursued the mechanisms involved. However, because the buffer can influence enzyme activity it is important to note what buffers are used when comparing results. Brains were solubilised in 0,5% Triton X-100/0,05M Tris-HCl, pH8,4 in all teratological experiments.

3.4.12. Determination of the effect of the concentration of Triton-X-100 on fetal mouse brain acetylcholinesterase activity

Introduction

The effect of varying the concentration of Triton X-100 on enzymatic activity has been commented on by various workers. Srinivasan et al (1972) using concentrations from 0,2 to 2% noted no alteration in enzyme activity, but when the concentration was increased to 5% and above, inhibition was demonstrable. Rieger and Vigny (1976) found the best solubilisation of AChE in brain tissues was obtained using an 0,5% to 1% Triton X-100 solution.

Method

Pooled 19 day fetal mouse brains (20mg/ml brain homogenate) were homogenised in 0,05M Tris-HCl containing varying concentrations of Triton X-100 (0 - 5%). The change in absorbance, Δ A, of each sample was determined spectrophotometrically (repeated x 3) and the AChE activity calculated in nmol/min/mg.

Results are given in Table 3.9. and shown graphically in Fig. 3.4.

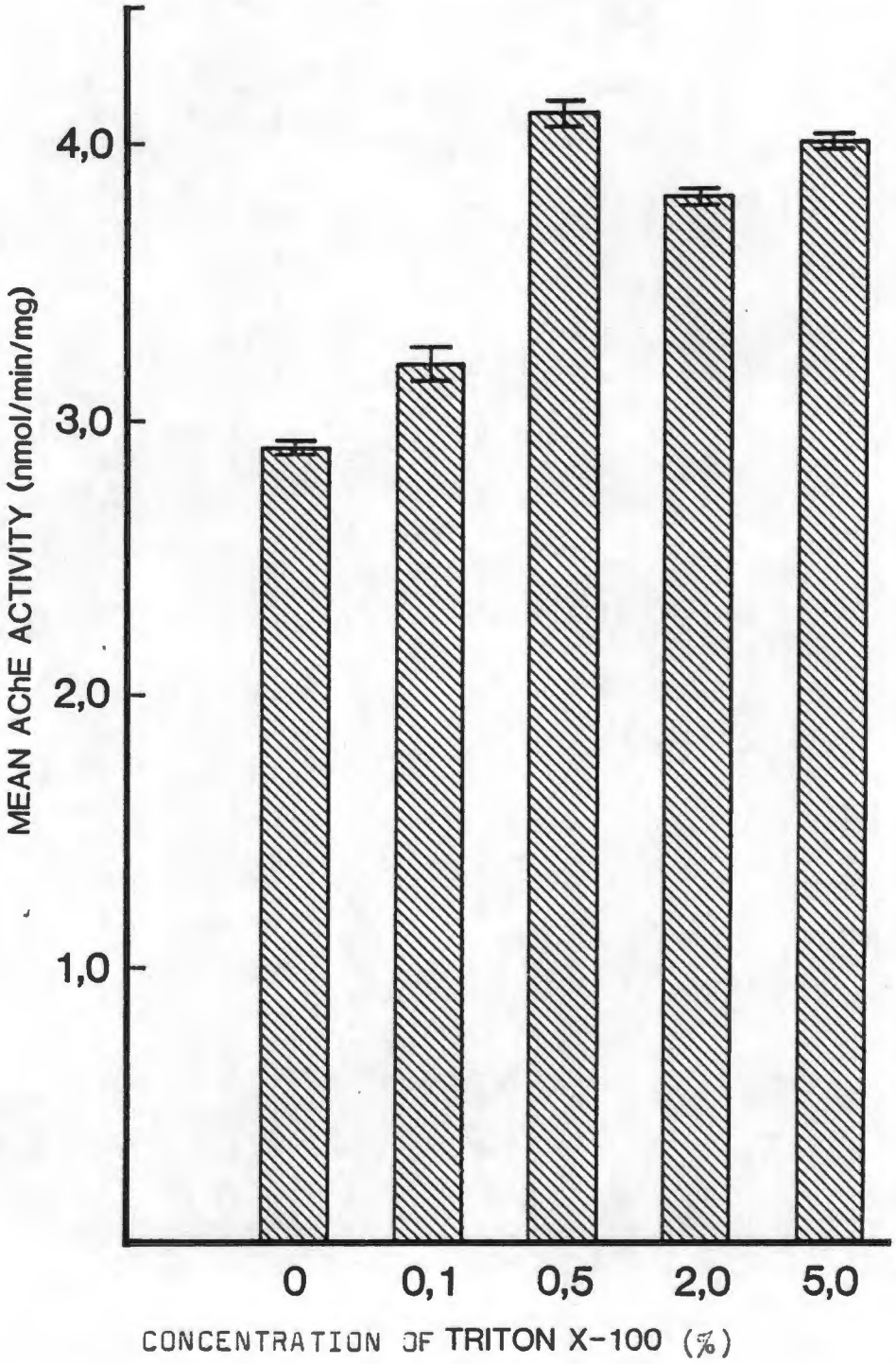
TABLE 3.9.

THE EFFECT OF THE CONCENTRATION OF TRITON X-100 ON FETAL MOUSE BRAIN ACETYLCHOLINESTERASE ACTIVITY

	Concentration of Triton X-100 (%)				
	0	0,1	0,5	2,0	5,0
AChE activity nmol/min/mg	2,871 2,888 2,936	3,328 3,165 3,132	4,111 4,046 4,176	3,850 3,850 3,768	4,046 4,013 3,964
MEAN	2,898	3,208	4,111	3,823	4,008
S.D.	0,0337	0,1049	0,0645	0,0473	0,0412

Fig. 3.4.

THE EFFECT OF THE CONCENTRATION OF TRITON X-100 ON FETAL MOUSE BRAIN AChE ACTIVITY



Error bars shown are observed scatter (3 observations)

Discussion and conclusion

With 0% and 0,1% Triton X-100 the brain homogenate remained turbid, and as shown in Table 3.9 and Fig. 3.4. the AChE activity was lower than that obtained with the higher concentrations of Triton. These observations are in keeping with inadequate solubilisation. The higher enzyme activity with 0,1% Triton X-100 compared with no Triton demonstrated, however, that some solubilisation occured even with this low concentration. With 0,5%, 2% and 5% Triton X-100 the brain homogenates were almost clear and higher enzyme activities were apparent. AChE activity was similar with the higher concentrations suggesting that maximal solubilisation occured with a concentration of 0,5% Triton. With 2% and 5% Triton no change in AChE activity was demonstrable.

It was therefore apparent that a concentration of Triton from 0,5% to 5% was suitable. Two percent Triton X-100 was used in earlier experiments and 0,5% in all subsequent experiments.

3.5. DETERMINATION OF THE CONTRIBUTION OF ACETYLCHOLINESTERASE ACTIVITY IN BLOOD TO TOTAL BRAIN ACETYLCHOLINESTERASE ACTIVITY

3.5.1. Introduction

A problem in performing quantitative measurements of brain AChE is that blood is inevitably present and blood is rich in both AChE in the red cells and cholinesterase in the serum. It is important to know the contribution of this enzyme activity in blood to total brain AChE activity. If the contribution is substantial, any changes observed in "brain" AChE could in fact be due to alterations in blood enzyme activity. Unfortunately, on reviewing the literature, it was apparent that this aspect

had not been taken into account when performing AChE assays on brain. Maletta and Timiras (1966) actually measured total esterase activity of blood when looking at brain AChE activity in developing rats after X-irradiation, but did not consider the contribution of this blood esterase to the results they obtained in brain.

A method was therefore devised for determining the contribution of the enzyme activity in blood to total brain AChE activity.

3.5.2. Materials and Methods

The principle of the method was to determine the amount of haemoglobin in blood and brain using haemoglobin concentration as an index of the amount of blood present. Acetylcholinesterase activity in blood and brain was then measured and the contribution of blood calculated.

Eighteen day C3H mouse fetuses were sacrificed by decapitation. Blood was collected from the decapitation wound into dry test tubes, weighed and homogenised in 0,5% Triton X-100, using a Thomas Teflon pestle homogeniser at 1200 rev/min for 1 minute. Brains were removed whole within 2 min of death, weighed, and homogenised in 0,5% Triton X-100. Homogenates were not centrifuged as a clear pink homogenate was obtained.

Haemoglobin determination was performed using the cyanmethaemoglobin method (van Kampen and Zijlstra, 1965; Drabkin and Austin, 1932, 1935). One Aculute diluent pellet (Orthadiagnostics, Raritan, New Jersey. Active ingredients : sodium bicarbonate, potassium ferricyanide, potassium cyanide) was dissolved in 250ml distilled water ("Drabkin's reagent"). 200µl of blood or brain homogenate was added to 3ml of Drabkin's reagent, mixed well and allowed to stand for 10 min to permit

cyanmethaemoglobin to form. The absorbance was then read at 540nm in a spectrophotometer (540nm is the light absorption maximum of hemiglobincyanide (HiCN) against a blank containing 200µl of 0,5% Triton X and 3ml Drabkin's reagent. This technique takes into account the fact that the haemoglobin solutions may contain the following substances : heamoglobin, oxyhaemoglobin, carboxyhaemoglobin, hemiglobin and other haemoglobin derivatives. All of these are converted to HiCN with Drabkin's reagent.

Acetylcholinesterase activities in blood and brain were determined colorimetrically by measuring the rate of hydrolysis of the substrate acetylthiocholine with a Cary Varian Spectrophotometer (see section 3.6.) The final reaction mixture consisted of 3ml 0,1 M-phosphate buffer pH 8,0; 200µl homogenate, 100µl DTNB (0,01 M) and 50µl acetylthiocholine iodide (0,075 M). Absorbance was determined at 410nm against a blank. Enzyme activity was expressed as nmol/min/mg to tissue.

3.5.3. Results

The results of absorbance at 540nm and AChE activity of brain and blood from six 18 day C3H mouse fetuses are presented in Tables 3.10 and 3.11.

Substantial blood enzyme activity is apparent (median AChE activity 1,71 nmol/min/mg) which is 52,8% that of brain (3,24 nmol/min/mg). The absorbance of haemoglobin (more correctly HiCN) at 540nm in brain is 1/16 that in blood. The contribution of blood to enzyme activity in brain is calculated as follows: Median brain weight = 85,6mg; median blood absorbance = 0,037; median blood weight = 24,63mg. For 1 mg blood, the absorbance is 0,037/24,63. For 85,6mg blood, the absorbance is 85,6 x (0,037/24,63) = 0,129. For 85,6mg brain, absorbance is 0,008. For an equal weight of blood, the absorbance is 0,129/0,008 = 16,13 x

TABLE 3.10.

WEIGHT, ABSORBANCE AND ACETYLCHOLINESTERASE ACTIVITY OF BRAIN SAMPLES FROM SIX 18-DAY C3H MOUSE FETUSES

BRAIN

Mouse No.	Wt. (mg)	Absorbance (at 540nm)	AChE activity (nmol/min/mg)
1	85,60	0,0130	4,53
2	85,80	0,0095	3,088
3	79,39	0,0076	2,74
4	88,73	0,0072	3,35
5	94,20	0,0080	3,26
6	75,20	0,0042	3,32
Median	85,60	0,0080	3,24
95% Confidence Limits	80,50 - 88,73	0,0101 - 0,0068	3,04 - 3,80

TABLE 3.11.

WEIGHT, ABSORBANCE AND ACETYLCHOLINESTERASE ACTIVITY OF BLOOD SAMPLES FROM SIX 18-DAY C3H MOUSE FETUSES

BLOOD

Mouse No.	Wt. (mg)	Absorbance (at 540nm)	Enzyme activity (nmol/min/mg
1	19,40	0,0341	1,82
2	21,30	0,0352	1,84
3	23,20	0,0364	1,86
4	29,76	0,0369	1,38
5	28,66	0,0400	1,57
6	26,05	0,0468	1,58
Median	24,63	0,0370	1,70
95% Confidence Limits	22,25 - 27,36	0,0355 - 0,0410	1,57 - 1,83

greater than absorbance of brain. Alternatively, the contribution of blood to brain or amount of blood in brain is 1/16,13. But the median blood AChE activity is 1,71 nmol/min/mg. Therefore, the contribution of blood to enzyme activity is 1,71/16,13 = 0,106 nmol/min/mg, compared with 3,24 nmol/min/mg mean brain AChE activity. Expressed as a percentage the contribution of AChE activity in blood to total brain AChE activity is 0,106 x 100/3,24 = 3,3%.

3.5.4. Discussion and Conclusion

In this experiment decapitation resulted in maximal drainage of blood from the brain and presumably accounts for the low contribution of blood to brain AChE activity. The same method of sacrifice was used throughout in this thesis. The amount of blood remaining in fetal brains was therefore likely to be similar and the contribution was accepted as representing an insignificant constant and was not determined independently in each assay.

CHAPTER 4

CHOLINESTERASE

4.1. INTRODUCTION

Cholinesterase (ChE, EC 3.1.1.8) is widely distributed in the central nervous system. The enzyme is located in white matter, principally in the supportive glial cells, fibrous astrocytes and Schwann cells of myelinated nerves (Koelle, 1950; 1952; Cavanagh et al., 1954; Mayer, 1980). In the cerebral white matter of man ChE activity exceeds that of AChE (Ord and Thompson, 1952). Studies with glioma tissue indicate that tumours of the astrocyte series in man are rich in ChE (Cavanagh et al.,1954). Although the enzyme has been shown histochemically to be present in the walls of blood vessels of brain (Koelle, 1952) this is not thought to contribute significantly to the activity in brain (Cavanagh et al.,1954). The physiological role in the brain is unknown but the association of ChE with the white fibre tracts of the brain has led to the suggestion that the enzyme is concerned with the metabolism of the myelin sheath or of neuroglial elements (Maletta and Timiras, 1965).

The aim of this study was to determine the ChE activity in C3H fetal mouse brains, before investigating the effect of teratogens on the enzyme. Four methods were used in the determination of cholinesterase activity utilising the following:

a) the preferred substrate of ChE; b) a selective ChE inhibitor; c) a selective AChE inhibitor; d) polyacrylamide gel electrophoresis.

4.2. DETERMINATION OF CHOLINESTERASE ACTIVITY USING THE PREFERRED SUBSTRATE, BUTYRYLTHIOCHOLINE IODIDE (BuThCh)

The preferred substrate of ChE is butyrylcholine (BuCh) or butyrylthiocholine (Silver, 1974:7). (In contrast AChE activity towards BuThCh is very low).

Materials and Methods

A. Pooled 18-day C3H fetal mouse brains were solubilised and homogenised as described in section 2.8. to give a brain concentration of 20mg/ml.

B. Brain homogenate was assayed for

a) Acetylcholinesterase activity using 50µl of 0,075M AThCh, as described in section 3.4.2. and

b) Cholinesterase activity using 0,037M BuThCh (Bonham et al., 1981). Volumes of components and the technique used were the same as those used for AChE assay, as described in section 3.4.2.

C. Each experiment was repeated three times. The rate of hydrolysis of ChE and AChE in brain homogenate was calculated as described in section 3.4.3. and the mean results are given in Table 4.1.

Results

TABLE 4.1.

ACTIVITY OF ACETYLCHOLINESTERASE AND CHOLINESTERASE IN U/l USING THE SUBSTRATES ACETYLTHIOCHOLINE AND BUTYRYLTHIOCHOLINE

SAMPLE	SUBSTRATE	
	AThCh	BuThCh
Brain homogenate	87,44	3,26

Comment

The enzyme activity of brain homogenate using BuThCh as substrate is approximately 3,7% ($\frac{3,26}{87,44}$ x 100) of that using AThCh as substrate, suggesting that a very small amount of ChE is present in fetal brain compared with AChE.

Some of this hydrolysis of BuThCh may be caused by AChE as BuThCh is not a totally specific substrate for ChE. (This was confirmed in a subsequent experiment using a specific AChE inhibitor - see section 4.4.

4.3. DETERMINATION OF CHOLINESTERASE ACTIVITY USING THE SELECTIVE CHOLINESTERASE INHIBITOR LYSIVANE (ETHOPROPAZINE HYDROCHLORIDE)

4.3.1. Introduction

The selective inhibitor of ChE, lysivane (ethopropazine hydrochloride or 10-(α-diethylaminopropyl) phenothiazine hydrochloride, May and Baker, South Africa) at a working concentration of 1.42×10^{-5} M has been found to produce 94.2% inhibition of ChE whilst producing 5.8% inhibition of AChE. (Dale et al., 1977; Dale et al., 1981). However, both the concentration of lysivane as well as the amount of enzyme present influence the degree of inhibition of the enzymes (Brock, 1981; Hullin and Elder, 1981).

Prior to determining cholinesterase activity the optimal concentration of lysivane to be used with C3H fetal mouse brain homogenates was therefore calculated (i.e. that concentration which gave maximum inhibition of ChE with minimum inhibition of AChE).

4.3.2. Preliminary experiments to determine the optimal concentration of lysivane using pure AChE (bovine) and ChE (serum)

Materials and Methods

A. 0,2mg pure bovine AChE (Sigma, 0,72 U/mg) was dissolved in 1,0ml distilled water.

B. Human serum provided a source of ChE.

C. Pure AChE and human serum were assayed for enzyme activity using the Ellman technique as described in section 3.4.2.

D. 50µl of stock lysivane solution was added to the test cuvette after allowing the initial reaction with substrate to proceed for 2 - 3 min. The change in the rate of hydrolysis was calculated.

A range of concentrations of lysivane were used (see Table 4.2.) to determine that concentration which gave the maximum inhibition of ChE with the minimum inhibition of AChE.

E. The percentage inhibition of enzyme activity was calculated by subtracting the rate of hydrolysis after addition of lysivane (y) from the original rate of hydrolysis (x) and dividing the difference by the original rate of hydrolysis. i.e.

$$\% \text{ inhibition} = \frac{x - y}{x} \times 100$$

$$\% \text{ remaining activity} = \frac{y}{x} \times 100$$

Results are shown in Table 4.2. and are plotted graphically in Fig. 4 .1.

TABLE 4.2.

RESULTS SHOWING : i) THE PERCENTAGE INHIBITION OF PURE BOVINE ACETYLCHOLINESTERASE AND ii) THE PERCENTAGE REMAINING ACTIVITY OF HUMAN SERUM CHOLINESTERASE WITH INCREASING LYSIVANE CONCENTRATION

LYSIVANE CONCENTRATION x 10^{-4} M (STOCK CONC.)	% INHIBITION OF PURE AChE	% REMAINING ACTIVITY OF SERUM ChE
4,5	2,29	5,95
6,8	4,25	4,65
9,12	4,38	4,09
13,7	7,32	3,62
18,2	9,27	3,47
27,4	16,13	2,77
36,4	15,74	2,35

a) A progressive increase in lysivane concentration caused an increase in the inhibition of both AChE and ChE activity.

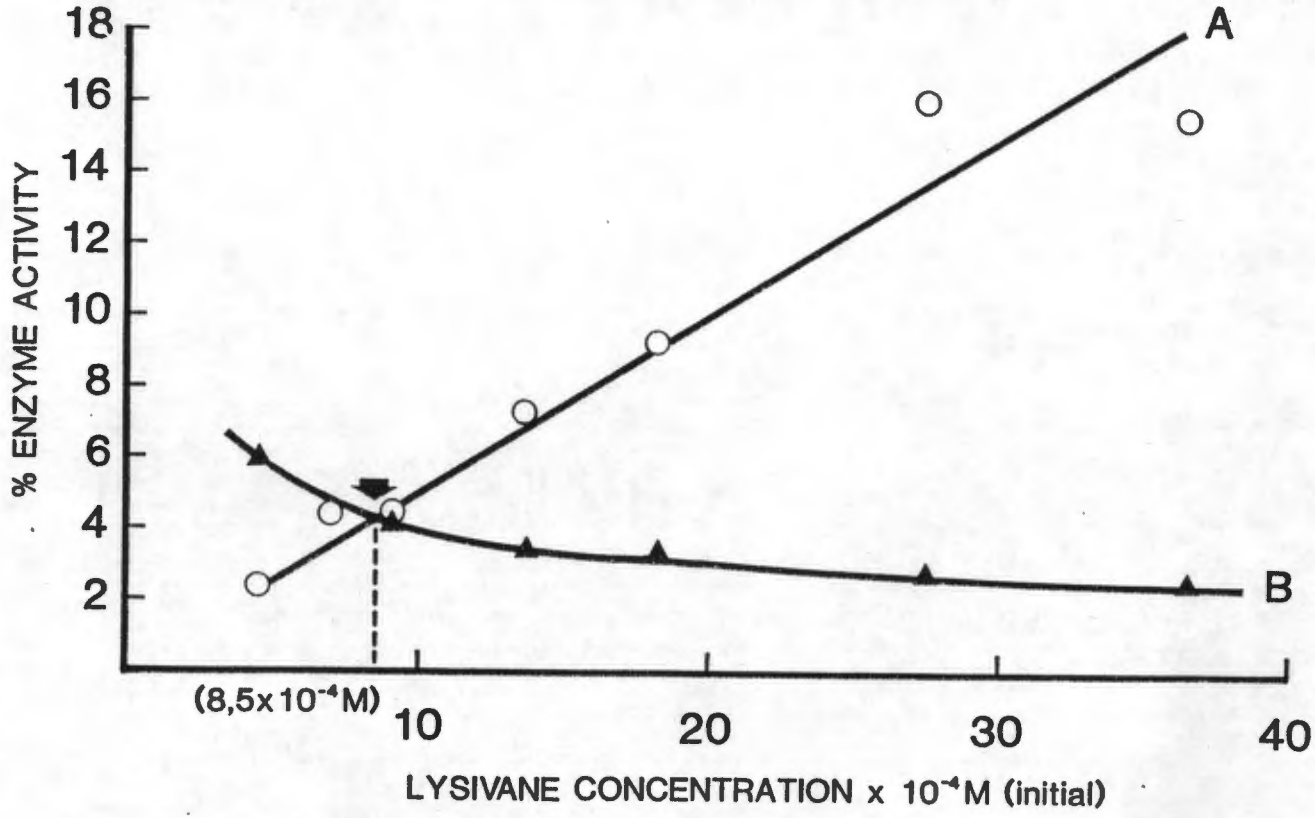
b) From Fig. 4.1. the intercept of lines A and B indicates that the optimal concentration of lysivane was 8,5 x 10^{-4}M producing 4,3% inhibition of pure bovine AChE and 95,7% inhibition of serum ChE.

4.3.3. Determination of the optimal concentration of lysivane using fetal mouse brains and human serum, and estimation of brain ChE

The previous experiment was repeated using fetal brains to

i) determine whether the optimal concentration of lysivane as obtained for pure AChE was the optimal concentration for

Fig. 4.1. PERCENTAGE INHIBITION OF PURE BOVINE AChE (A) AND PERCENTAGE REMAINING ACTIVITY OF HUMAN SERUM ChE (B) WITH INCREASING LYSIVANE CONCENTRATION



fetal brain (20mg/ml brain homogenate)

ii) to estimate the ChE activity in fetal mouse brain.

Materials and Methods

A. Pooled 18 day C3H mouse brains were solubilised and homogenised as described in section 2.8. to give a brain concentration of 20mg/ml.

B. Human serum was used as a source of ChE.

C. Brain homogenate and human serum were assayed and the percentage inhibition of enzyme activity calculated as in section 4.3.2.

D. Different concentrations of lysivane were used (see Table 4.3.) to determine that concentration which gave maximum inhibition of ChE with minimum inhibition of AChE.

Results and discussion

Results are given in Table 4.3. and plotted graphically in Fig. 4.2.

a) A similar graph to that obtained in the previous experiment was obtained. The intercept of lines C and D also gives an optimal lysivane concentration of $8,5 \times 10^{-4}$M.

b) With the optimal lysivane concentration of $8,5 \times 10^{-4}$M the percentage inhibition of total brain esterase (i.e. AChE and ChE) was 4,6%. However, this concentration of lysivane produced 4,3% inhibition of AChE in the previous experiment, suggesting that only 0,3% of this total % was ChE.

c) The lines for (i) the percentage inhibition of brain AChE and ChE and (ii) the percentage remaining activity of ChE, have been drawn to best fit the available points and therefore may be approximate. However, this is unlikely to have caused more than a few percent error in the above deductions.

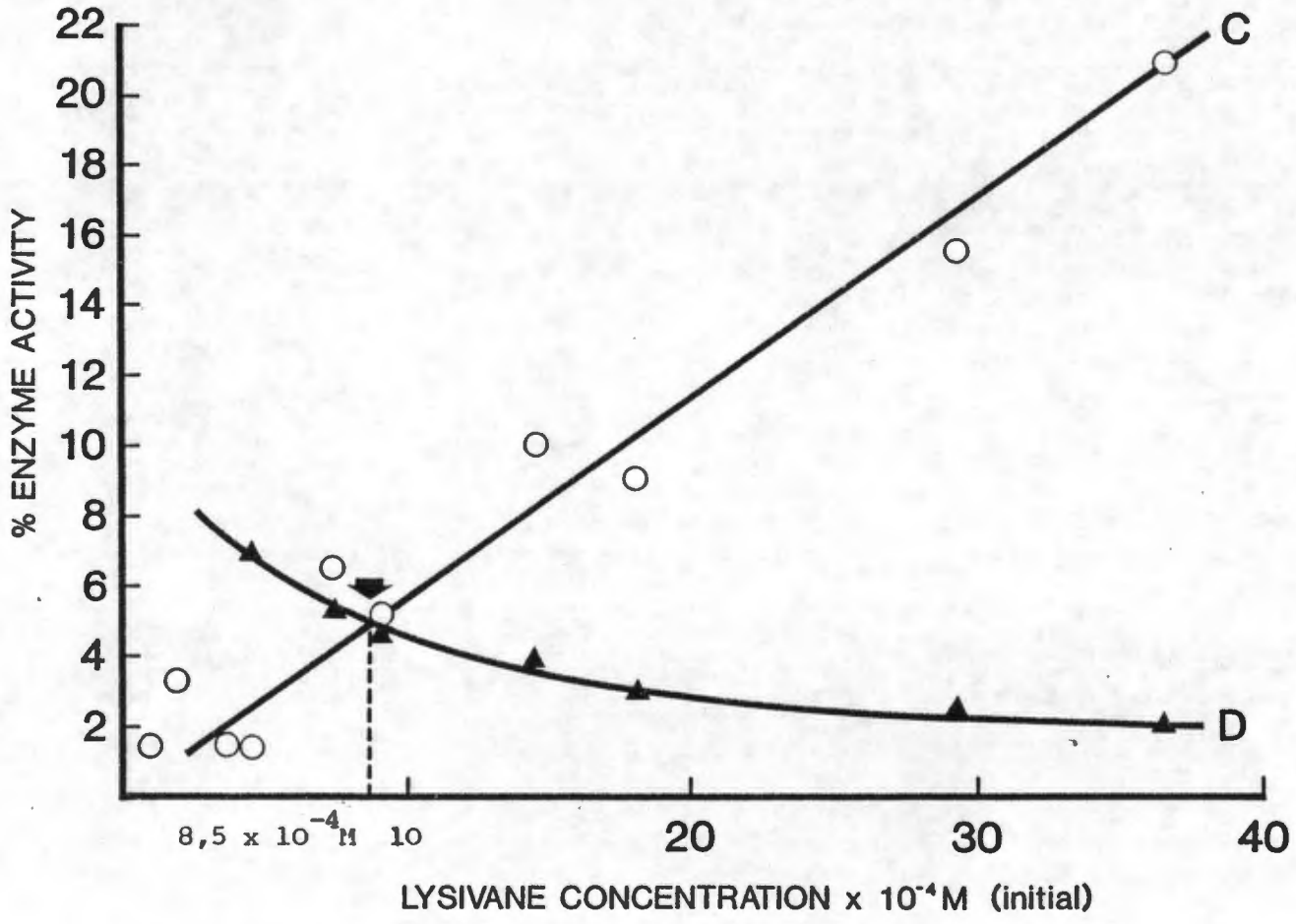
T A B L E 4.3.

RESULTS SHOWING : i) THE PERCENTAGE INHIBITION OF BRAIN ACETYLCHOLINESTERASE AND ii) THE PERCENTAGE REMAINING ACTIVITY OF HUMAN SERUM CHOLINESTERASE WITH INCREASING LYSIVANE CONCENTRATION

LYSIVANE CONC. x10^{-4}M (STOCK CONC.)	% INHIBITION OF BRAIN AChE	% REMAINING ACTIVITY OF SERUM ChE
1,14	1,46	*
1,83	3,27	*
3,67	1,56	*
4,56	1,38	6,95
7,33	6,59	5,64
9,12	5,18	4,66
14,7	10,01	3,86
18,2	9,2	3,03
29,3	15,47	2,46
36,5	20,79	2,05

* not assayed

Fig. 4.2. PERCENTAGE INHIBITION OF FETAL MOUSE BRAIN AChE AND ChE AND PERCENTAGE REMAINING ACTIVITY OF HUMAN SERUM ChE (D) WITH INCREASING LYSIVANE CONCENTRATION



4.4. DETERMINATION OF CHOLINESTERASE ACTIVITY USING THE SELECTIVE ACETYLCHOLINESTERASE INHIBITOR, 1,5-BIS (4 ALLYLDIMETHYL AMMONIUM PHENYL)-PENTAN-3-ONE DIBROMIDE (B.A.P.)

Acetylcholinesterase can be distinguished from ChE by the use of a so-called "specific" inhibitor such as B.A.P. which inhibits AChE but not ChE (Silver, 1974:9). Using the Ellman technique as described in section 3.4.2., if AChE activity in fetal brain homogenate is inhibited by the selective inhibitor B.A.P., any remaining enzyme activity should be due to ChE.

Materials and Methods

A. Pooled 18-day C3H fetal mouse brains were solubilised and homogenised as described in section 2.8. to give a brain concentration of 20mg/ml.

B. The brain homogenate was assayed for enzyme activity using a) 0,075M AThCh and b) 0,037M BuThCh as substrates as described in section 3.4.2. and 4.2.

C. 50µl of 0,0125M B.A.P. (this was found to be a suitable concentration from preliminary experiments) was added to the cuvette after allowing reaction with substrate for 2 - 3 min.

D. The rate of hydrolysis before and after addition of B.A.P. was calculated. Each experiment was performed three times.

Results and discussion

Results (the mean of 3 assays) are given in Table 4.4.

TABLE 4.4.

ENZYME ACTIVITY IN BRAIN HOMOGENATE BEFORE AND AFTER ADDITION OF B.A.P.

SUBSTRATE	ENZYME ACTIVITY nmol/min/mg	ENZYME ACTIVITY AFTER ADDITION OF B.A.P. nmol/min/mg	% INHIBITION
AThCh	4,37	0,297	93,20
BuThCh	0,163	0,132	19,02

a) When AThCh was used as the substrate, addition of the selective AChE inhibitor to the reaction mixture resulted in 93,2% inhibition of enzyme activity, demonstrating that most of the enzyme activity in brain was due to AChE.

b) The relative amount that ChE activity contributed to the total enzyme activity (AChE and ChE) in brain homogenate was determined in two ways:

i) using AThCh as substrate, the remaining enzyme activity after the addition of B.A.P. was calculated and expressed as a percentage of the total enzyme activity:

$\frac{0,297}{4,37} \times 100 = 6,8$ % of total brain enzyme activity.

ii) using BuThCh as substrate, the remaining enzyme activity after the addition of B.A.P. was calculated and expressed

as a percentage of total enzyme activity using AThCh as substrate = $\frac{0,132}{4,37} \times 100 = 3,0\%$

i.e. calculated in this way 3% of the total brain esterase activity was due to ChE (i.e. the relative amount that ChE contributed to total brain esterase activity was small.)

The discrepancy between this latter result of 3% and the former result of 6,8% is probably explained by the fact that enzyme activities in the latter calculation were compared using different substrates.

c) Using BuThCh as substrate, the 19% inhibition of enzyme activity after addition of B.A.P., demonstrates that some of the hydrolysis of BuThCh is due to AChE, as B.A.P. is a selective AChE inhibitor.

4.5. ATTEMPTS TO DEMONSTRATE THE PRESENCE OF CHOLINESTERASE IN FETAL MOUSE BRAIN USING POLYACRYLAMIDE GEL ELECTROPHORESIS

If significant amounts of ChE are present in 18 day C3H fetal mouse brain, this should be demonstrable as a distinct zone on polyacrylamide gel electrophoresis, as for example in human amniotic fluid (Seller and Cole, 1980; Smith et al, 1979). Attempts were made to demonstrate ChE in fetal mouse brain by

i) incubating a gel with the preferred ChE substrate BuThCh, and

ii) by comparing a control gel incubated with AThCh as substrate with gels incubated with AThCh and

a) the ChE inhibitor, lysivane and

b) the AChE inhibitor, B.A.P.

Materials and Methods

A. Polyacrylamide gels were prepared as described in Chapter 5.

B. 18-day C3H fetal mouse brains were solubilised and homogenised as described in section 2.8.

C. To confirm that a ChE band could be demonstrated on the gels, human serum (as a source of ChE) was used in a separate well. Fetal mouse serum was also used to demonstrate the position of fetal mouse ChE on the gel.

D. Pure bovine AChE (Sigma) was run in a separate well (250µl of a 0,2mg/ml AChE solution was mixed with 150µl of sample buffer).

E. Samples for electrophoresis were prepared as follows:

i) 400µl of brain homogenate was used with 80µl of a glycerol/ sucrose solution and 20µl bromophenol blue (tracking dye) - see Chapter 5.

ii) 100µl human serum or fetal mouse serum was mixed with 300µl sample buffer (0,05M Tris-HCl, 0,5% Triton X-100, pH 8,4); 80µl glycerol/sucrose and 20µl bromophenol blue.

F. Eight µl samples were loaded into separate wells of the gel with a Hamilton syringe.

G. Electrophoresis was performed at 20mA/gel for approximately 4h. (A full description of the electrophoresis is given in Chapter 5).

H. Gels were incubated as follows:

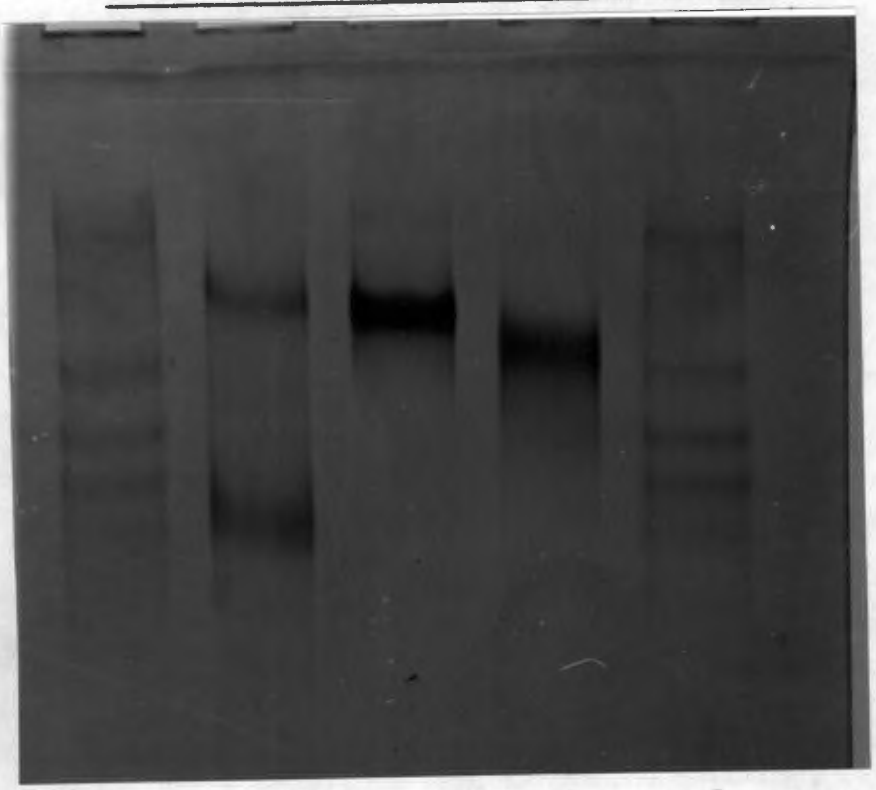
a) with AThCh as substrate only (0,116mg/100ml) (see Fig. 4.3.)

b) with AThCh as substrate and lysivane (1,5ml of 8,85 x 10^{-4} stock in 100 ml) (see Fig. 4.4.)

Fig. 4.3.

POLYACRYLAMIDE GEL

INCUBATION WITH ACETYLTHIOCHOLINE IODIDE



TRACK 1 2 3 4 5

Key:

Tracks 1 and 5 : 18-day fetal mouse brain homogenate

Track 2 : 18-day fetal mouse serum

Track 3 : Human serum

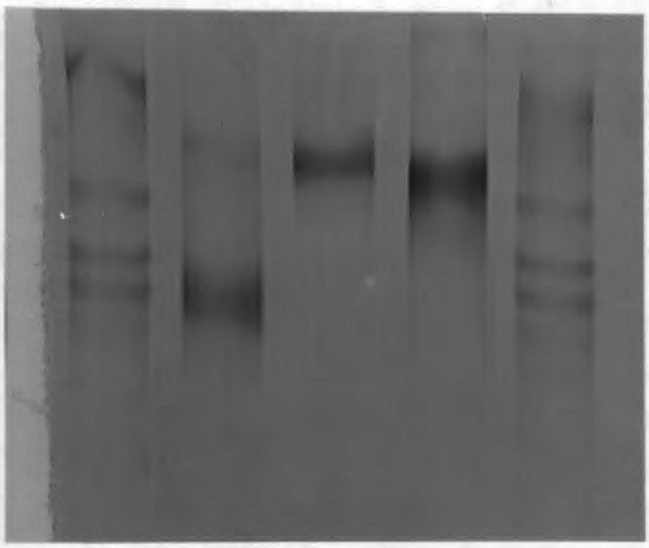
Track 4 : Pure bovine AChE

Note:

- the typical 5 band pattern of C3H fetal mouse brain
- the 2 bands in fetal mouse serum
- the single band in human serum
- the single band with pure bovine AChE.

Fig. 4.4.

POLYACRYLAMIDE GEL - INCUBATION WITH ACETYLTHIOCHOLINE IODIDE AND LYSIVANE



TRACK 1 2 3 4 5

Key: As for Fig. 4.3.

Compare with Fig. 4.3.

Note:

- the marked decrease in the intensity of the slow moving band in fetal serum (track 2) and the band in human serum (track 3), confirming that these are ChE.
- no decrease in intensity of pure bovine AChE (track 4) or of fetal brain bands (tracks 1 and 5).

c) with AThCh as substrate and B.A.P. (0,028g in 100ml, 5 x 10^{-4} final M) (see Fig. 4.5.)

d) with BuThCh as substrate only (0,116mg/100ml) (see Fig. 4.7. and compare with AThCh as substrate, Fig. 4.6.)

Results

i) with lysivane there was no demonstrable change in the fetal brain isoenzyme bands (compare tracks 1 and 5 in Fig. 4.3. and 4.4.), although very significant inhibition was apparent with serum. The fetal mouse serum ChE band was almost completely inhibited (track 2, Fig. 4.4.) and there was a decreased staining intensity of the human serum ChE band (track 3, Fig. 4.4.) (see note b). This suggested that the isoenzyme bands in fetal brain were all AChE with no ChE demonstrable. Alternatively, a ChE band could have been obscured by an AChE band and was therefore not apparent.

ii) with the selective AChE inhibitor B.A.P. complete inhibition of all fetal mouse brain isoenzyme bands occured (track 3, Fig. 4.5.), revealing that there was no demonstrable residual ChE band. The bovine AChE band was also completely inhibited (track 1, Fig. 4.5.) The slow-moving ChE band in mouse fetal serum was not inhibited whereas the fast moving AChE band was (track 3, Fig. 4.5.) (see note "a"). Similarly, the ChE band in human serum was not inhibited (track 4, Fig. 4.5.)

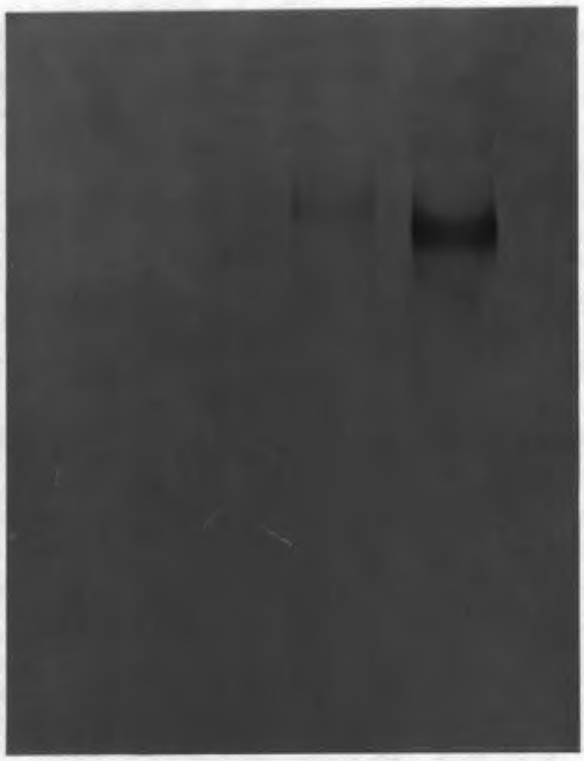
iii) with the preferred substrate BuThCh, ChE activity could also not be demonstrated in fetal mouse brain (Fig. 4.7.) even though a ChE band was well demonstrated with human serum (track 3, Fig.4.7.)

Note:

a) Some AChE was present in mouse fetal serum. i.e. the fast

Fig. 4.5.

POLYACRYLAMIDE GEL: INCUBATION WITH ACETYLTHIOCHOLINE IODIDE AND B.A.P.



TRACK 1 2 3 4

Key:

Track 1: pure bovine AChE
Track 2: 18 day fetal mouse brain homogenate
Track 3: 18 day fetal mouse serum
Track 4: human serum

Note:

- in track 1 no band is visible (compare track 4 in Fig. 4 .3. incubated without B.A.P.) showing complete inhibition of AChE.

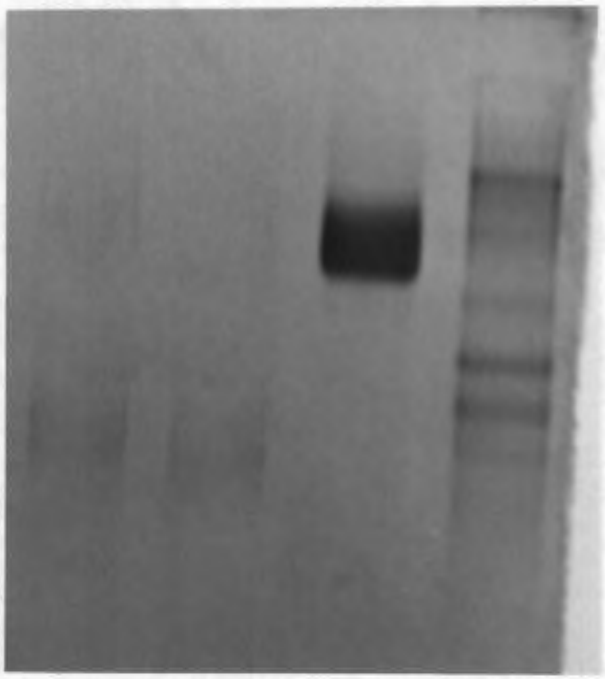
- in track 2 all the 5 brain bands have disappeared (compare tracks 1 and 5 in Fig. 4 .3. without B.A.P.) suggesting these are AChE.

- in track 3 the fast moving band of fetal mouse serum has disappeared (compare track 2 in Fig. 4 .3. without B.A.P.) suggesting this is AChE.

- in track 4 there is no inhibition of the band (compare track 3 in Fig. 4 .3.) demonstrating no inhibition of ChE.

Fig. 4.6.

POLYACRYLAMIDE GEL: INCUBATION WITH ACETYLTHIOCHOLINE IODIDE



TRACK 1 2 3 4

Key:

Tracks 1 and 2: mouse amniotic fluid

Track 3 : human serum

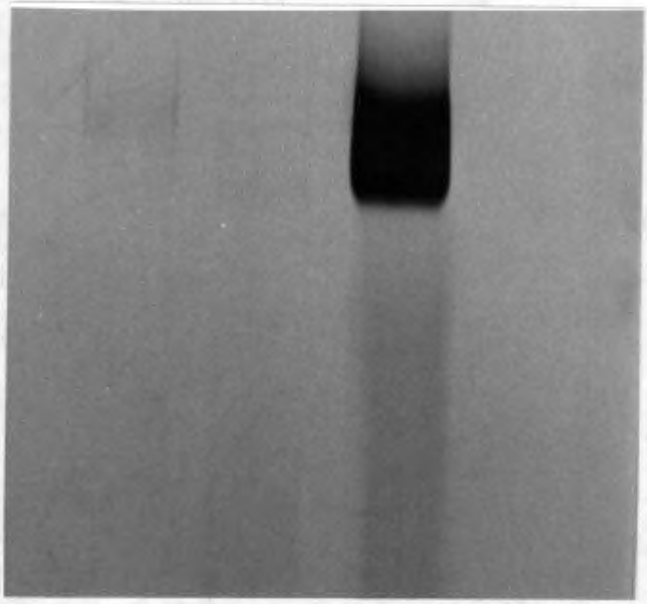
Track 4 : 18 day fetal mouse brain homogenate

Note: (COMPARE WITH FIG. 4.7.)

- the typical 5 band pattern of C3H fetal mouse brain (see Chapter 6)
- the faint fast-moving band of AChE in amniotic fluid
- the dark slow-moving band in human serum

Fig. 4.7.

POLYACRYLAMIDE GEL : INCUBATION WITH BUTYRYLTHIOCHOLINE



TRACK: 1 2 3 4

Key:

Tracks 1 and 2: mouse amniotic fluid

Track 3 : human serum

Track 4 : 18-day fetal mouse brain homogenate

Note: (Compare with Fig. 4.6.)

- the faint slow-moving band in amniotic fluid (tracks 1 and 2) - demonstrating the small amount of ChE in amniotic fluid
- the very dark ChE band in human serum (track 3) showing the high concentration of ChE
- no ChE band in fetal brain (track 4) (The 5 AChE bands are not apparent when incubated with BuThCh.)

moving band which was not inhibited by lysivane (track 2, Fig. 4.4.) This was probably due to some red cell contamination of serum as it was impossible with the quantities available to separate pure serum.

b) Complete inhibition of human serum ChE was not achieved presumably because of the high concentration of ChE present for the relatively low concentration of lysivane used (track 3, Fig. 4.4.)

4.6. DISCUSSION AND CONCLUSIONS

a) Cholinesterase activity in 18-day C3H fetal mouse brain is very low, contributing only 3 - 6,7% to the total enzyme activity (AChE and ChE), depending on the technique used. With polyacrylamide gel electrophoresis of fetal mouse brains, no ChE activity could be demonstrated.

As far as I can ascertain there are no studies in the literature on fetal mouse brain ChE activity. However, the above findings are consistent with those of other workers with the postnatal rat and mouse. In adult rat whole brain the activity of ChE in hydrolysing butyrylcholine has been found to be approximately 6% of that of AChE in hydrolysing acetylcholine (Woolley, 1963). Ellman (1961) found the average rate of hydrolysis of butyrylthiocholine by adult rat brain was 3% that of the average rate of hydrolysis of acetylthiocholine. Cholinesterase activity in C3H adult mouse brains was found to be 2% of AChE activity (Pryor, 1966) whilst in C57BL/6 and DNA/2 mice at birth ChE activity was 8% of AChE activity (Pryor, 1968). With polyacrylamide gel electrophoresis of 30-day-old rat brain esterases Vijayan and Brownson (1974) were only

able to demonstrate a single weakly reactive cholinesterase isoenzyme in the brain stem.

b) Due to the low ChE activity, the effect of teratogens on its activity was not assessed.

c) Because the contribution of ChE to total "AChE" activity was very small, and would have been approximately the same in different brains, it was accepted as representing an insignificant constant and was not determined independently for each assay nor subtracted from the AChE activity.

d) The low ChE activity is further support for the very small amount of residual blood in fetal brain with the decapitation technique used. (Blood serum is rich in ChE).

CHAPTER 5

SEPARATION OF THE ISOENZYMES OF FETAL MOUSE BRAIN ACETYLCHOLINESTERASE : POLYACRYLAMIDE GEL ELECTROPHORESIS

5.1. INTRODUCTION

Several investigators have reported varying numbers of acetylcholinesterase isoenzymes in rat and mouse brain. In the rat brain Vijayan and Brownson (1974) detected 3 isoenzymes of AChE on polyacrylamide gel electrophoresis. Each of the 3 zones was noted to consist of several subzones on densitometry. The fastest moving band was designated isoenzyme one and the slowest moving band isoenzyme three. Zone three exhibited maximum staining and accounted for more than 50% of the total enzyme activity. Comparison of the isoenzyme patterns of serum, liver, skeletal muscle and intestine revealed that brain alone exhibited a marked preponderance of isoenzyme three. Although AChE activity is known to vary in different parts of the brain, it was noted that the relative percentage values for each isoenzyme remained constant in the various regions. There was also no difference between males and females, and the three isoenzymes responded to inhibitors in a similar way. Using a similar technique Cambon et al., (1980) separated 3 isoenzymes from fetal and maternal rat brain. Also using polyacrylamide gel electrophoresis, Bajgar (1979) found 4 isoenzymes in the supernatant of rat brain homogenate, and noted different thermal stabilities of the isoenzymes. With microgel gradient electrophoresis 3 to 4 AChE zones were noted during post natal development in the rat brain (Bischoff et al.,1982).

Wenthold et al.,(1974) observed six isoenzymes in rat brain using electrofocusing, whereas Ho and Ellman (1969), after treatment with bacterial protease observed three different forms of AChE in rat brain. In the adult mouse, Adamson et al.,(1975) noted two major zones of enzyme activity after polyacrylamide gel electrophoresis of fresh extracts of brain. A third zone which migrated more slowly was noted in aged extracts. It is apparent that the number of isoenzymes detected varies with different workers, the experimental technique used, and the species.

Several workers have suggested that the isoenzymes appear to correspond to aggregates (Bernsohn et al.,1963; Chan et al.,1972; Adamson et al.,1975; Wenthold et al.,1974), whilst others have noted differences in the physico-chemical and enzymatic properties of the isoenzymes such as the Km and pH optima (Bajgar and Zizkovsky, 1971), or differences in the 3 forms in bovine brain (Chan et al.,1972) or of thermal stabilities (Bajgar, 1979), suggesting they are indeed isoenzymes.

As far as I can ascertain from the literature the AChE isoenzymes of fetal mouse brain have not been characterised. The first aim of this study, which is addressed in this chapter, was to establish a satisfactory technique for the separation of the isoenzymes of AChE. Subsequent studies to investigate the effects of three teratogens, vitamin A in large doses, cyclophosphamide and sodium valproate, on these isoenzymes are presented under the relevant chapters. The potential of the isoenzymes as markers of teratogenic injury was considered because teratogens can alter normal development and may be toxic to the CNS. Also the possibility exists that the isoenzymes may exhibit differential sensitivity to embryotoxins. Alterations in AChE isoenzymes have been reported

during the normal maturation of the nervous system (Iqbal and Talwar, 1971). Similarly, Rieger and Vigny (1976) found an increase in one AChE isoenzyme relative to another in rat brains during development. Alterations in AChE isoenzymes have also been demonstrated in experimental brain injury in the absence of a significant change in total AChE levels (Lim et al., 1972). Differential AChE isoenzyme inhibition has been noted with parathion (Vijayan and Brownson, 1975) and in rat fetuses after maternal carbamate poisoning (Cambon et al., 1980).

Polyacrylamide gel electrophoresis of the isoenzymes of AChE was performed on vertical slab gels (Raymond, 1964; Allen and Moore, 1966) utilizing a non-dissociating continuous Tris-glycine buffer system. Under non-dissociating buffer conditions, electrophoresis results in retention of biological activity of the isoenzymes (Hames, 1981 : 7). Separation is on the basis of size and charge of the molecules (Chrambach and Rodbard, 1971). The technique used was based on that of Allen and Moore (1966), Vijayan and Brownson (1974) and Haddow (1981) and modified. Significant detail has been included so that the procedure can be easily followed. The lack of relevant technical detail in the literature on the separation of the isoenzymes of AChE resulted in many months of trial and error before satisfactory separation and resolution could be obtained.

5.2. MATERIALS AND METHODS

A. Preparation of gels

A 7,5% gel mixture was prepared as follows: 10ml 30% Acrylogel (BDH biochemicals), 10ml gel buffer (0,05M Tris, 0,386 M glycine, pH 8,4), 20ml distilled H_2O, 0,2ml Triton X-100 and 20µl Temed (catalyst : N,N,N′,N′ - Tetramethylethylenediamine were stirred on a magnetic stirrer until the Triton had dissolved. Just prior to

pouring 200µl of 10% ammonium persulphate (initiator) was added.

The mixture was poured between two glass plates, dimensions 16cm x 14cm, 1,5mm apart. A leak-proof seal was obtained by using a thin layer of Celloseal between the bottom of the glass plates and the rubber washer of the casting stand. Using a Pasteur pipette the gel mixture was overlaid with water (to help exclude oxygen which inhibits polymerisation). Polymerisation occurred in 45 min, giving a clear or faintly opaque gel. After 1h the layer of water was decanted off and a second 7,5% gel (prepared as above except that 300µl ammonium persulphate and 40µl Temed were used) poured on top of the gel. Sample wells (25mm deep, volume 300µl) were placed in the second gel by means of a 10 well perspex comb inserted at the top of the glass plates. Polymerisation of the second gel took approximately 15 min and the comb was removed after 45 - 60 min. The wells were washed with reservoir buffer.

B. Sample preparation (brain)

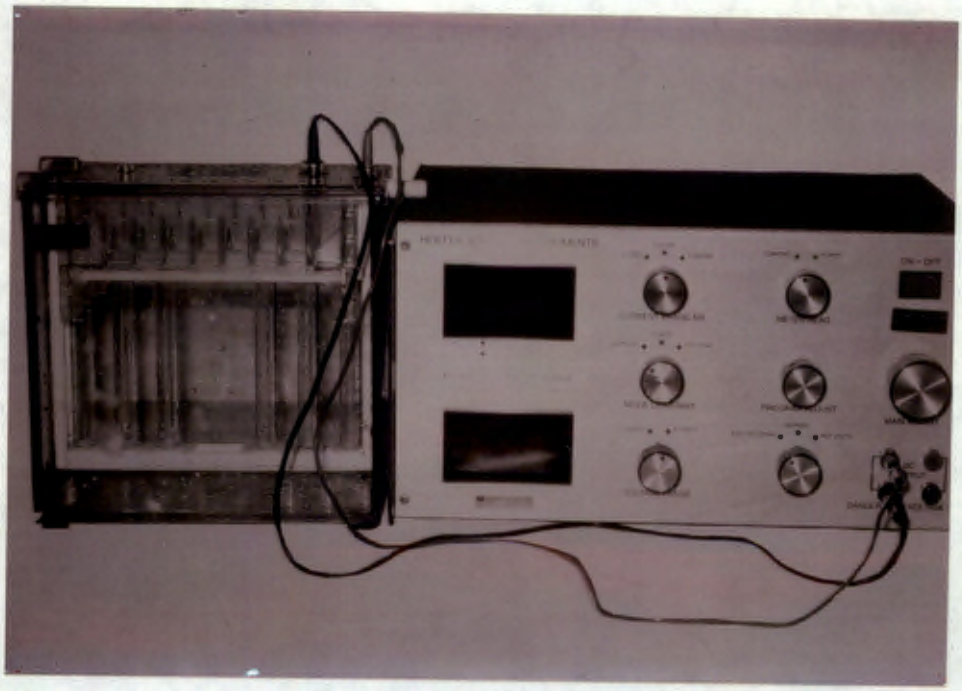
Fetal brains (20mg per ml brain homogenate) were solubilised and homogenised as described in section 2.8. To 100µl of homogenate was added 20µl of 1:1 glycerol-water/85% sucrose mixture and 5µl of tracking dye (bromophenol blue).

C. Electrophoresis

A Hoefer SE 600-10-1.5 vertical slab electrophoresis unit and a PS 1200 power supply unit (Hoefer) were used (Fig. 5.1). Bubbles were removed from the bottom of the gel as these prevent uniform electrical contact between gel and reservoir buffer. Tris-glycine buffer (0,01M Tris, 0,077M glycine, pH 8,4) was used in both the lower and upper tanks. Seven microlitre samples were carefully layered into the sample wells with a Hamilton 1701N syringe. Electrophoresis was performed at a constant current of 20mA per gel for

Fig. 5.1.

HOEFER ELECTROPHORESIS EQUIPMENT



Note:

- the Hoefer SE 600-10-1.5 vertical slab electrophoresis unit and the PS 1200 power supply unit.

3 - 3½h at which stage the marker dye was 4cm from the bottom of the gel. Starting voltage was ± 300 volts and final voltage after 3h was ± 670 volts. During electrophoresis the temperature was maintained between 18 and 20°C by circulating water at 18 to 20°C continuously through the heat exchanger.

D. Enzyme development and staining

The development medium was based on that of Haddow (1981). After electrophoresis the gel was placed as follows: a) In 100ml pre-incubation mixture (20g sodium sulphate (Na_2SO_4), 1,25g maleic acid, 0,15g glycine, 0,1g copper sulphate ($CuSO_4.5H_2O$) and 0,06g magnesium chloride ($MgCl_2.6H_2O$), adjusted to pH 6.5 with 6M sodium hydroxide) for 30 min;

b) in 100ml incubation medium containing 116mg acetylthiocholine iodide as substrate in 100ml pre-incubation solution for 20h;

c) in post-incubation mixture (20g sodium sulphate (Na_2SO_4) and 1,25g maleic acid, adjusted to pH 6.5 with 6M sodium hydroxide) for 30 min before thoroughly washing with distilled water. (If thorough washing was not carried out blotchiness of the background gel could result which posed problems for densitometric analysis).

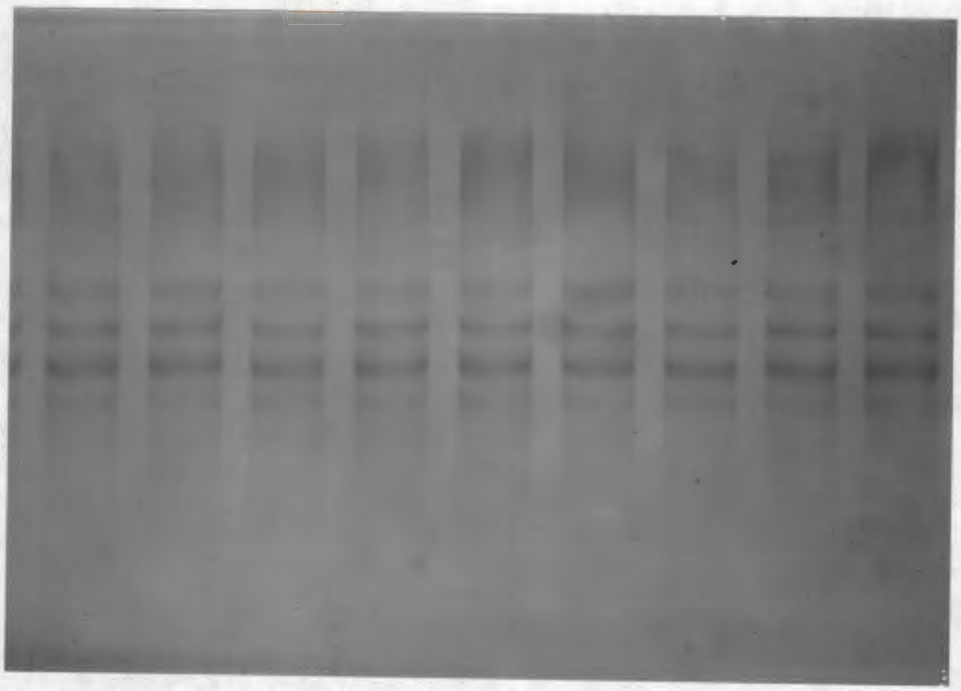
For better visualisation of the isoenzymes the gels were stained with a saturated solution of dithio-oxamide for 2h. The isoenzymes showed up as dark green-black bands against a uniform faint green background.

5.3. RESULTS

Figure 5.2. is a photograph of a typical polyacrylamide gel demonstrating the AChE isoenzymes of 19-day C3H fetal mouse brains.

Fig. 5.2.

POLYACRYLAMIDE GEL DEMONSTRATING THE AChE ISOENZYMES OF 19-DAY FETAL MOUSE BRAINS



Note:

- each track is from a different fetus.
- the five distinct zones.
- the bands are numbered 1 to 5, band 1 being the fastest moving, rather faint zone at the bottom and band 5 the slowest moving diffuse zone.
- bands 2 and 3 stain most intensely.

Five distinct bands are apparent and are numbered 1 to 5, band 1 being the fastest moving, rather faint zone and band 5 the slowest moving diffuse zone. Bands 2, 3 and 4 were well resolved and of these bands 2 and 3 had the highest activity.

5.4. SOME PRACTICAL POINTS AND PROBLEMS

5.4.1. Polyacrylamide gels

A. Slab gels

Slab gels were used for the following reasons (Hames, 1981 : 5):

a) slab gels enabled direct side by side comparison of samples;

b) heat produced during electrophoresis was easily dissipated, thus reducing distortion of bands due to heating effects; c) their rectangular cross section allowed densitometry and photography with little risk of optical artifacts; d) a large number of samples could be electrophoresed under identical conditions.

B. Choice of gel concentration

Running gel

The best separation and resolution of isoenzymes was obtained using a 7,5% uniform gel (see Fig. 5.2.) There was no advantage in using a gradient gel. A 4,5 - 9% gradient gel, which gave the best results of various concentration gradients used, also yielded comparable quality bands to those of the 7,5% gel, except that with the 7,5% gel 5 distinct bands were evident as opposed to only 3 with the gradient gel. Also, the 7,5% gel gave more reproducible results.

"Stacking" gel

Using a large pore stacking gel, e.g. 4,5%, there was no improvement

in resolution of the bands. A 7,5% gel was therefore used. This was layered onto the resolving gel as the higher concentration of ammonium persulphate needed to give adequate polymerisation with the perspex combs in position can result in streaking if incorporated in the resolving gel.

C. Acrylogel versus bis-acrylamide (NN'-methylene bisacrylamide) and acrylamide

There was no advantage using bis-acrylamide and acrylamide as opposed to Acrylogel. Acrylogel (BDH biochemicals) was used in all definitive experiments.

D. Inclusion of Triton X-100 in the gel

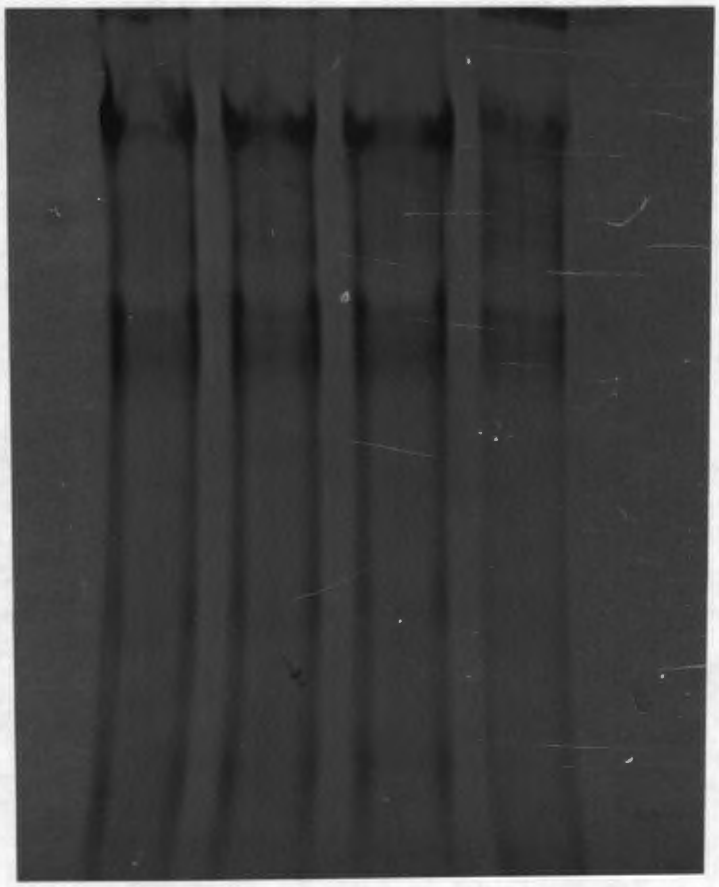
Triton X-100 was included in the gel at the same concentration (0,5%) as that used for solubilisation. Very poor results were obtained if Triton was omitted from the gel, such as bizarre separation and aggregation of the isoenzymes (Fig. 5.3.)

E. "Wavy Lines"

Wavy transparent lines were commonly seen at the tops of gels, no doubt related to the higher oxygen concentration at the top of the gel which is closest to the atmosphere. The oxygen interferes with polymerisation and leads to uneven pore formation. The wavy lines result in uneven wavy bands of isoenzymes. Degassing both the water layered on the top of the gels and the gel mixture did not solve the problem. However, by choosing the appropriate gel concentration the bands move well down into the gel away from the wavy lines.

Fig. 5.3.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF FETAL MOUSE BRAIN HOMOGENATE : OMISSION OF TRITON X-100 FROM THE GEL



Note:

- the absence of separation of isoenzyme bands and the bizarre aggregation.

5.4.2. Buffers

Choice of buffer system

Using continuous or discontinuous buffer systems quoted in the literature (e.g. Haddow, 1981; Clarke, 1964; Vijayan and Brownson, 1974) I was unable to obtain satisfactory separation and resolution of isoenzymes. After much trial and error the following buffer system was selected: a continuous low ionic Tris/glycine buffer system (gel buffer: 0,05M Tris, 0,39M glycine, pH 8,4; reservoir buffer: 0,01M Tris, 0,077M glycine, pH 8,4).

Sample buffer

In preliminary experiments the same Tris/glycine buffer was used as in the gel. There was no difference in the quality of the isoenzyme bands with either Tris-glycine or Tris-HCl and a 0,05M Tris-HCl buffer, pH 8,4 was used in all definitive experiments. At lower ionic strengths e.g. 0,01 - 0,03M Tris-HCl, aggregation of enzyme occurred resulting in streaking.

5.4.3. Electrophoresis

A. Pre-electrophoresis

This was found to be unnecessary, i.e. there was no difference in the separation and resolution of the bands compared with non-pre-electrophoresed gels.

B. Electrophoresis with constant current versus constant voltage

Electrophoresing with constant voltage as opposed to constant current

gave poorer results, presumably because the longer time required allowed significant diffusion to occur.

C. Duration of electrophoresis

Three to 3½ hours was found to be the optimal duration for electrophoresis at 20 mA/gel (at this time the dye front was 3-4cms from the bottom of the gel). Shorter runs gave inadequate separations whilst protracted runs resulted in excessive diffusion with loss of resolution of the bands.

D. Temperature control during electrophoresis

Water was circulated continuously at 18 - 20°C through the cooling system during electrophoresis. Temperature must be controlled if electrophoretic separations are to be reproducible because heat is generated during electrophoresis and the mobility of migratory ions is increased as temperature increases (Hames, 1981 : 18).

5.4.4. Brain samples

A. Brain concentration

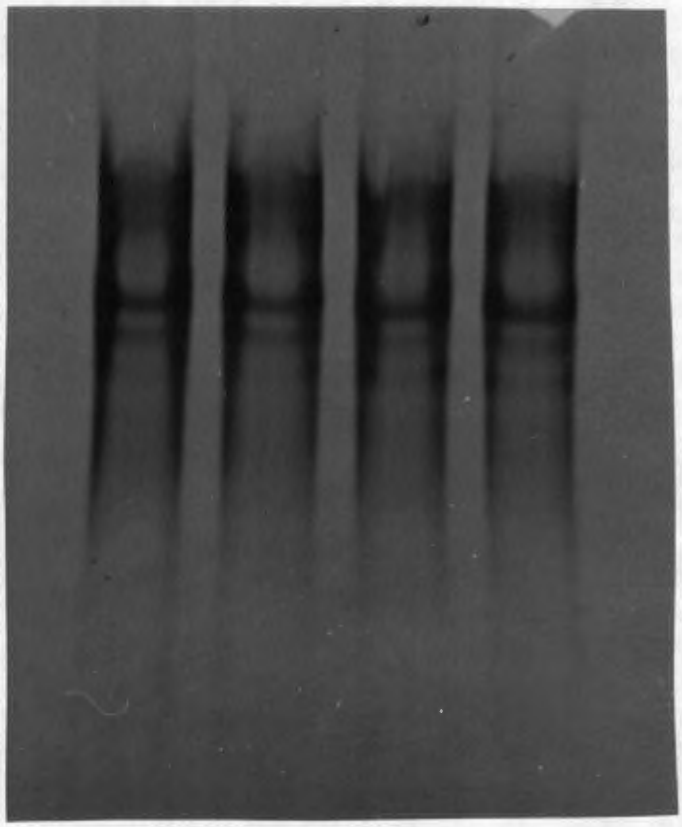
Good results were obtained using a brain concentration of 20mg/ml. With lower concentrations the bands were too faint. Above this concentration there was no significant improvement whilst adding the problem of minimal volumes of homogenate to work with.

B. Sample load

If sample loads were too large aggregation of enzyme occurred resulting in streaking (protein streaks running parralel to the direction of migration) (Fig. 5.4.) A 7µl sample load containing

Fig. 5.4.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF FETAL MOUSE BRAIN HOMOGENATE : EXCESSIVE SAMPLE LOAD RESULTING IN STREAKING



Note:

- the vertical streaks and aggregation of enzyme along the margins of the tracks from excessive sample loading.

20mg/ml of brain was chosen because this gave good visualization of isoenzymes with negligible streaking.

C. Centrifugation

Centrifugation of brain homogenates (e.g. at 8000g for 30 minutes) prior to electrophoresis did not result in any improvement in resolution or separation of the isoenzymes and was therefore not performed. This was an advantage as centrifugation would inevitably lead to some loss of enzyme in the centrifugate.

D. Sonication

Sonication offered no advantage over homogenisation and solubilisation with Triton X-100 nor did submitting the sample to both procedures.

5.5. QUALITY OF ISOENZYME SEPARATION

After overcoming the various problems detailed above the technique was reproducible with good resolution and separation of the isoenzymes of AChE (see Fig. 5.2.). Isoenzyme separation appeared superior to that published in the literature where photographic documentation of isoenzyme zones after polyacrylamide gel electrophoresis was given (e.g. Vijayan and Brownson, 1974; Seller and Cole, 1980). The bands were suitable for densitometric analysis.

The only poorly resolved zone was band 5 i.e. the diffuse slow moving first zone which was a constant feature and may have represented a poorly resolved isoenzyme. However, increasing the duration of the run did not resolve this zone into a distinct band. Alternatively, it may have been due to isoenzyme aggregation, or to the enzyme being caught up with protein moieties. Incorporating trypsin in the hope that this would digest possible protein moieties unfortunately resulted in

loss of all enzyme activity. Varying sample load or the ionic strength of sample buffer, centrifugation, or increasing the concentration of Triton in which the brains were solubilised made no demonstrable difference. It was not due to non-specific staining of non-enzyme substances as omitting the substrate acetylthiocholine iodide resulted in no staining.

5.6. INHIBITOR STUDIES

To confirm the composition of the isoenzyme bands inhibitor studies were performed.

A control gel incubated with AThCh as substrate (0,116mg/100ml) was compared with gels incubated with AThCh and a) the AChE inhibitor B.A.P. (0,028g/100ml = 5 x 10^{-4} final M) and b) the ChE inhibitor lysivane (1,5ml of 8,85 x 10^{-4}M stock in 100ml).

Human serum as a source of ChE was run in a separate well. Fetal mouse serum was run in another well to demonstrate the position of fetal mouse ChE, and pure bovine AChE in a further well.

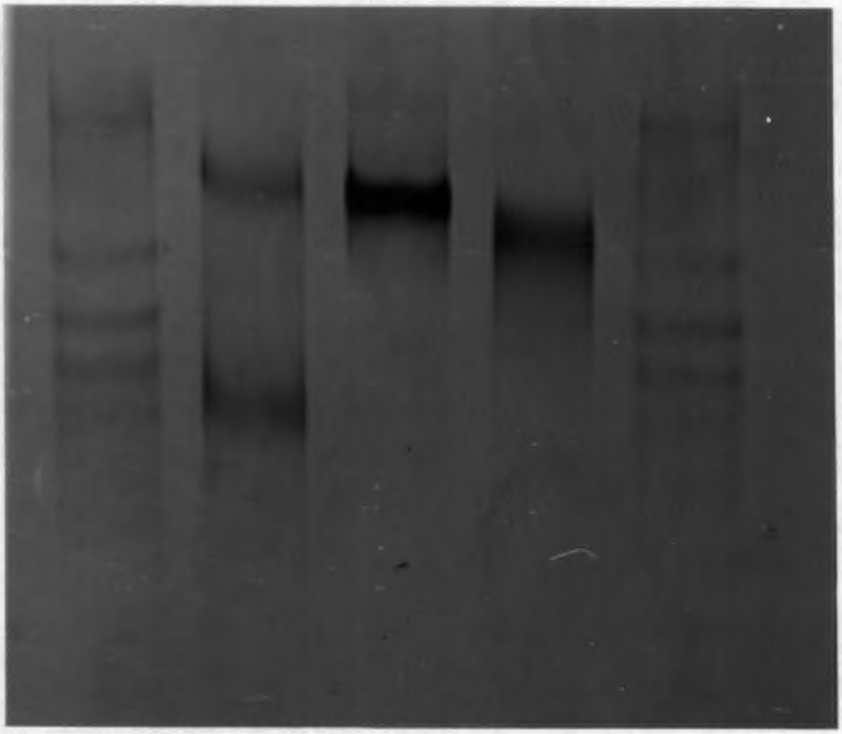
Results

With the selective AChE inhibitor B.A.P. complete inhibition of all fetal mouse brain isoenzyme bands occured (see Fig. 5.6. and compare with control gel, Fig. 5.5.), indicating that the bands were indeed AChE. Note that the ChE band in human and fetal mouse serum was not inhibited with B.A.P. (Fig. 5.6.) and that no inhibition of fetal mouse brain isoenzymes occured with lysivane (Fig. 5.7.), which was further support as stated in section 4.5., that the isoenzyme bands were AChE with no ChE demonstrable.

Fig. 5.5.

POLYACRYLAMIDE GEL

INCUBATION WITH ACETYLTHIOCHOLINE IODIDE



TRACK 1 2 3 4 5

Key:

Tracks 1 and 5 : 18-day fetal mouse brain homogenate

Track 2 : 18-day fetal mouse serum

Track 3 : Human serum

Track 4 : Pure bovine AChE

Note:

- the typical 5 band pattern of C3H fetal mouse brain
- the 2 bands in fetal mouse serum
- the single band in human serum
- the single band with pure bovine AChE.

Fig.5.6.

POLYACRYLAMIDE GEL: INCUBATION WITH ACETYLTHIOCHOLINE IODIDE AND B.A.P.

TRACK 1 2 3 4

Key:

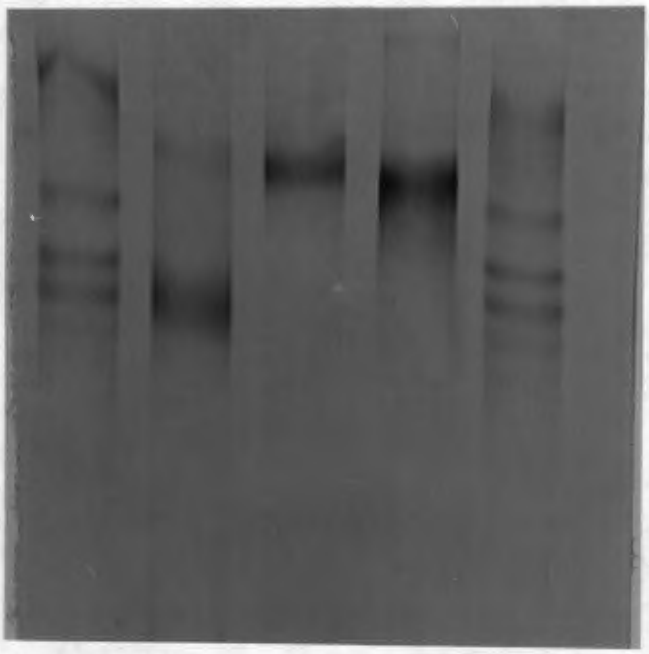
Track 1: pure bovine AChE
Track 2: 18-day fetal mouse brain homogenate
Track 3: 18-day fetal mouse serum
Track 4: human serum

Note:

- in track 1 no band is visible (compare track 4 in Fig. 5.5. incubated without B.A.P.) showing complete inhibition of AChE.
- in track 2 all the 5 brain bands have disappeared (compare tracks 1 and 5 in Fig. 5.5. without B.A.P.) suggesting these are AChE.
- in track 3 the fast moving band of fetal mouse serum has disappeared (compare track 2 in Fig. 5.5. without B.A.P.) suggesting this is AChE.
- in track 4 there is no inhibition of the band (compare track 3 in Fig. 5.5.) demonstrating no inhibition of ChE.

Fig. 5.7.

POLYACRYLAMIDE GEL - INCUBATION WITH ACETYLTHIOCHOLINE IODIDE AND LYSIVANE



TRACK 1 2 3 4 5

Key: As for Fig. 5.5.

Compare with Fig. 5.5.

Note:

- the marked decrease in the intensity of the slow moving band in fetal serum (track 2) and the band in human serum (track 3), confirming that these are ChE.
- no decrease in intensity of pure bovine AChE (track 4) or of fetal brain bands (tracks 1 and 5).

5.7. DENSITOMETRY

5.7.1. Introduction

Densitometry was chosen as a quantitative technique for comparing the activity in the various isoenzyme bands of AChE.

Densitometry (as opposed to other techniques for quantitative analysis of gel patterns) was chosen because it has been successfully used for similar purposes - the analysis of AChE isoenzymes in mouse brain (Adamson et al, 1975); rat brain (Srinivasan at al, 1976; Vijayan and Brownson, 1974) and amniotic fluid (Goldfine et al, 1983). Densitometry appears to be a sensitive technique for comparing the relative amounts of AChE in different isoenzymes and is a simple, rapid method which can be used to analyse a large number of samples in intact gels.

5.7.2. Principles of technique

Densitometry measures the relative density (stain intensity) of a band with respect to a reference or standard and the background and depends on the amount of light absorbed by the band or fraction.

Absorbance of light is described by the Beer-Lambert law where:

Absorbance or A = alc; (c = concentration of sample (moles/l;
l = light path length (cm); a = absorptivity).
From this equation, the concentration (c) of a sample can be determined directly.

In the case of an electrophoresis pattern, the whole pattern is scanned by the densitometer and a graph is produced, plotting the position of each fraction in the sample. The concentration of each fraction can

then be determined by calculating either the area under the respective peaks (total c of the sample and total area under curve) or the peak heights of the fractions.

5.7.3. Materials and methods

a) Each gel (wet) was placed onto a clean glass plate and covered with a sheet of transparent plastic. Any air bubbles present were removed. The plastic covering prevented the gels from drying out or being damaged during scanning and provided a smooth, even surface to the gel.

b) The gel was positioned on the scanning stage of the densitometer and the start and end points of the scan set.

c) The densitometer used was a Zeiss KM3 Chromatogram Spectrophotometer - set up for measuring transmission as opposed to reflectance. Gels were scanned at a wavelength of 610 nm, using a tungsten lamp (325-2500 nm) and a slit size of 8 mm x 0.1 mm (Note : the width of the isoenzyme bands was 8 mm).

d) Before scanning, a zero value was obtained by placing the measuring head over a blank region of the gel. Maximum peak height was obtained by placing the measuring head on the densest band of the gel.

e) Gels were scanned at a speed of 100 cm/min and the scan was recorded at 500 mV full scale deflection.

5.7.4. Densitometric analysis of 18-day C3H fetal mouse brain AChE isoenzymes

Fetal mouse brain homogenate was prepared as described in section 2.8. Separation of the isoenzymes of AChE by polyacrylamide gel electrophoresis was performed as described in Chapter 5.

Densitometry was performed as described in section 5.7.3. and a typical

trace is shown in Fig. 5.8.

Results and discussion

Five main peaks were visible on the densitometric scans and their positions corresponded well to the 5 bands on the gels (Fig. 5.8. and 5.9.).

Background noise tended to vary between samples on one gel and between individual gels. This variation in the background was probably due to uneven staining.

Individual peak heights and the sum of peak heights for each sample, were analysed statistically using the Mann Whitney U test (non parametric); modified Wilcoxen Median test and the Kalmogarov-Smirnov test.

5.7.5. Preliminary experiments

To determine (i) whether there was a linear relationship between stain intensity and AChE concentration; (ii) the sensitivity of the densitometer.

Methods

A. (a) Serial dilutions of pure bovine AChE (Sigma 0,72 Units/mg) were prepared as follows: 400; 200; 100; 50; 25; 12,5µg/ml.

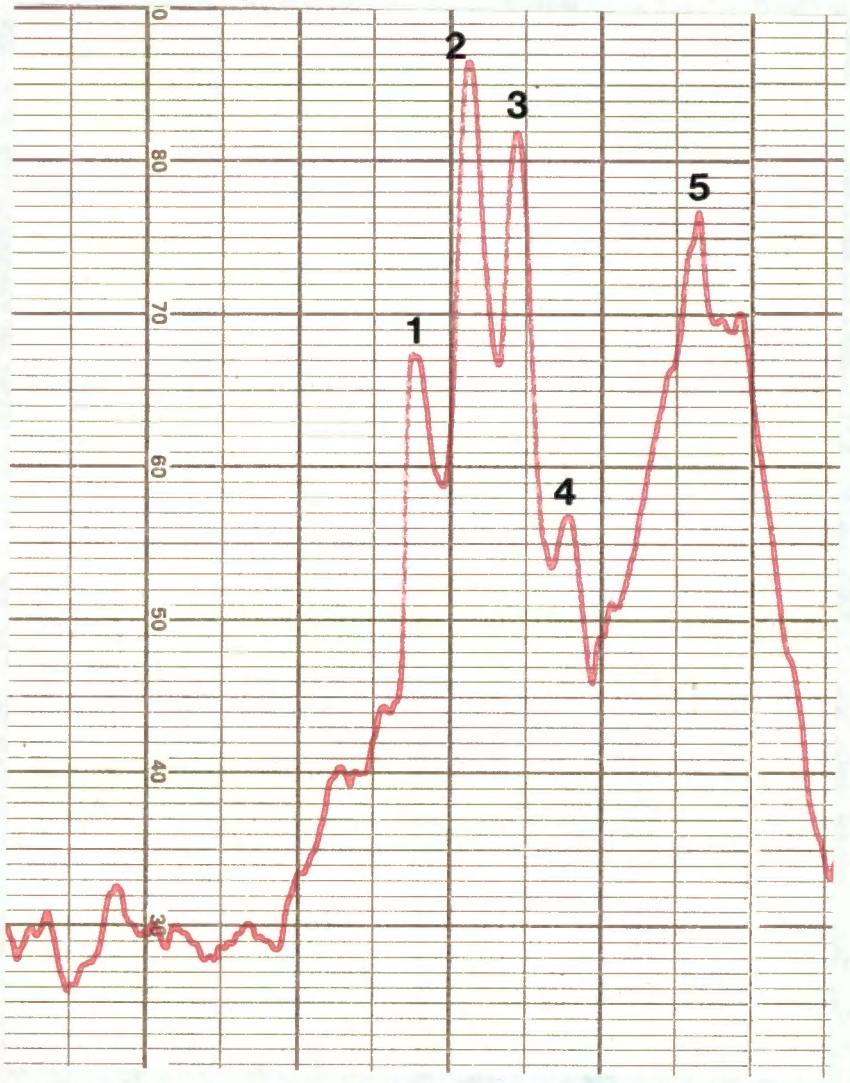
(b) 250µl of each AChE dilution was mixed with 150µl sample buffer (0,05M Tris-HCl; 0,5% Triton X-100, pH 8,4) and 20µl bromophenol blue (B.P.B.) and 80µl of glycerol/sucrose solution (sample loading buffer)

(c) 8µl of each sample was layered onto a 7,5% polyacrylamide gel.

Fig. 5.8.

DENSITOMETER TRACING OF AChE ISOENZYME PATTERN OF

19-DAY FETAL MOUSE BRAIN

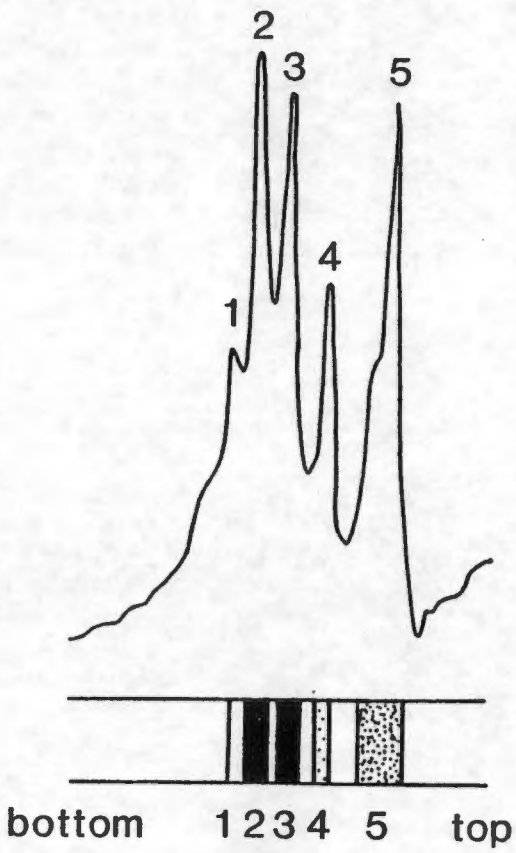


Note:

- the five isoenzyme peaks numbered 1-5

The peak heights for the 5 peaks were measured from an arbitrarily determined, best apparent baseline on each recorded scan. Peak heights were chosen as opposed to area under the curve (a.u.c.). Peak heights are acceptably accurate and were better suited to the tracings because of the difficulty in measuring a.u.c. for the following reasons: (i) there was high background noise; (ii) the peaks were not always smooth; and (iii) the troughs of some peaks did not return to baseline.

Fig. 5.9.



AChE ISOENZYMES OF 19-DAY C_3H FETAL MOUSE BRAINS

a) Densitometer tracing of the isoenzyme pattern.

b) Isoenzyme pattern on polyacrylamide gels.

B. (a) Eighteen day fetal mouse brain homogenate was prepared as described in section 2.8. (30mg/ml).

(b) Dilutions were made up as follows: 400; 300; 200; 100µl brain homogenate mixed with sample buffer to give a total volume of 400µl.

(c) 20µl of B.P.B. and 80µl of glycerol/sucrose solution was added to the above and 8µl of each sample was loaded onto a gel.

Electrophoresis was performed at 20 mA/gel for 4 hours. The gels were scanned on a Zeiss KM3 chromotagram spectrophotometer, at a wavelength of 510 nm and with a slit size of 8 mm x 0,1 mm. The densitometer scans were analysed by measuring the amplitude of the peaks.

Results and conclusions

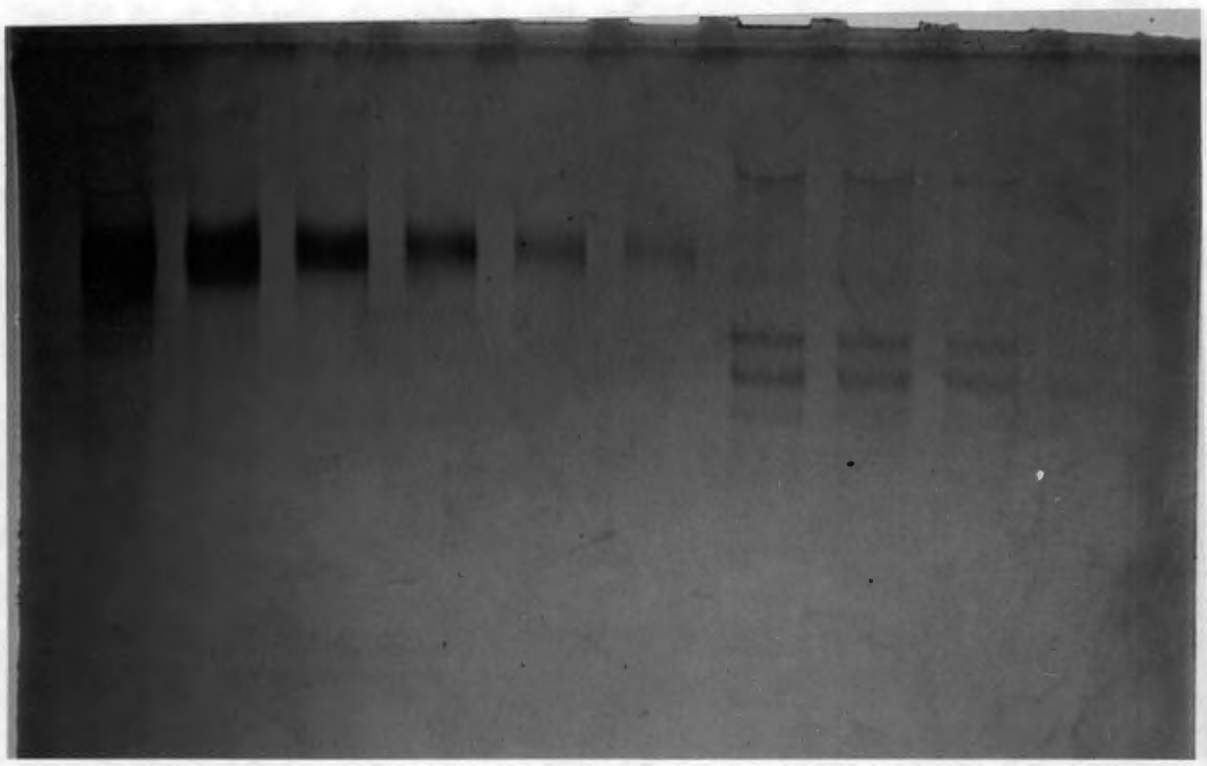
Figure 5.10. is a photograph of the gel showing the 6 serial dilutions of pure AChE (tracks 1 - 6) and the 4 serial dilutions of fetal mouse brain homogenate (tracks 7 - 10). With a dilution ratio of 0,25 (track 10) - the bands were too faint and were not subjected to densitometric analysis. The results of the relationship between pure AChE concentration and peak heights measured at 3 different sensitivities (+2V, +1V and +500 mV) on the densitometer are given in Table 5.1. and shown graphically in Fig. 5.11.

The results of the relationship between fetal mouse brain homogenate concentrations and peak heights measured at + 500 mV on the densitometer are given in Table 5.2. and shown graphically in Fig. 5.12.

There was a linear relationship between peak heights and AChE concentration at lower AChE concentrations (i.e. 0,05 - 0,2µg/8µl but not at concentrations above 0,2µg/8µl (see Fig. 5.11.) - this is inevitable given the absorptiometric nature of the assay .

Fig. 5.10.

POLYACRYLAMIDE GEL WITH SERIAL DILUTIONS OF PURE AChE AND 18-DAY FETAL MOUSE BRAIN HOMOGENATES



TRACK 1 2 3 4 5 6 7 8 9 10

Key:

Tracks 1-6: serial dilutions of pure AChE from 1,6 µg/8 µl to 0,05 µg/8 µl.

Tracks 7-10: serial dilutions of fetal mouse brain homogenates from 24 mg/ml to 6 mg/ml.

Note:

the stain intensity of "undiluted" brain homogenate (track 7) is less than that of 0,2 µg/8 µl of pure AChE (track 4).

Results

TABLE 5.1.

RELATIONSHIP BETWEEN PURE ACETYLCHOLINESTERASE - CONCENTRATION AND PEAK HEIGHTS MEASURED AT THREE DIFFERENT SENSITIVITIES

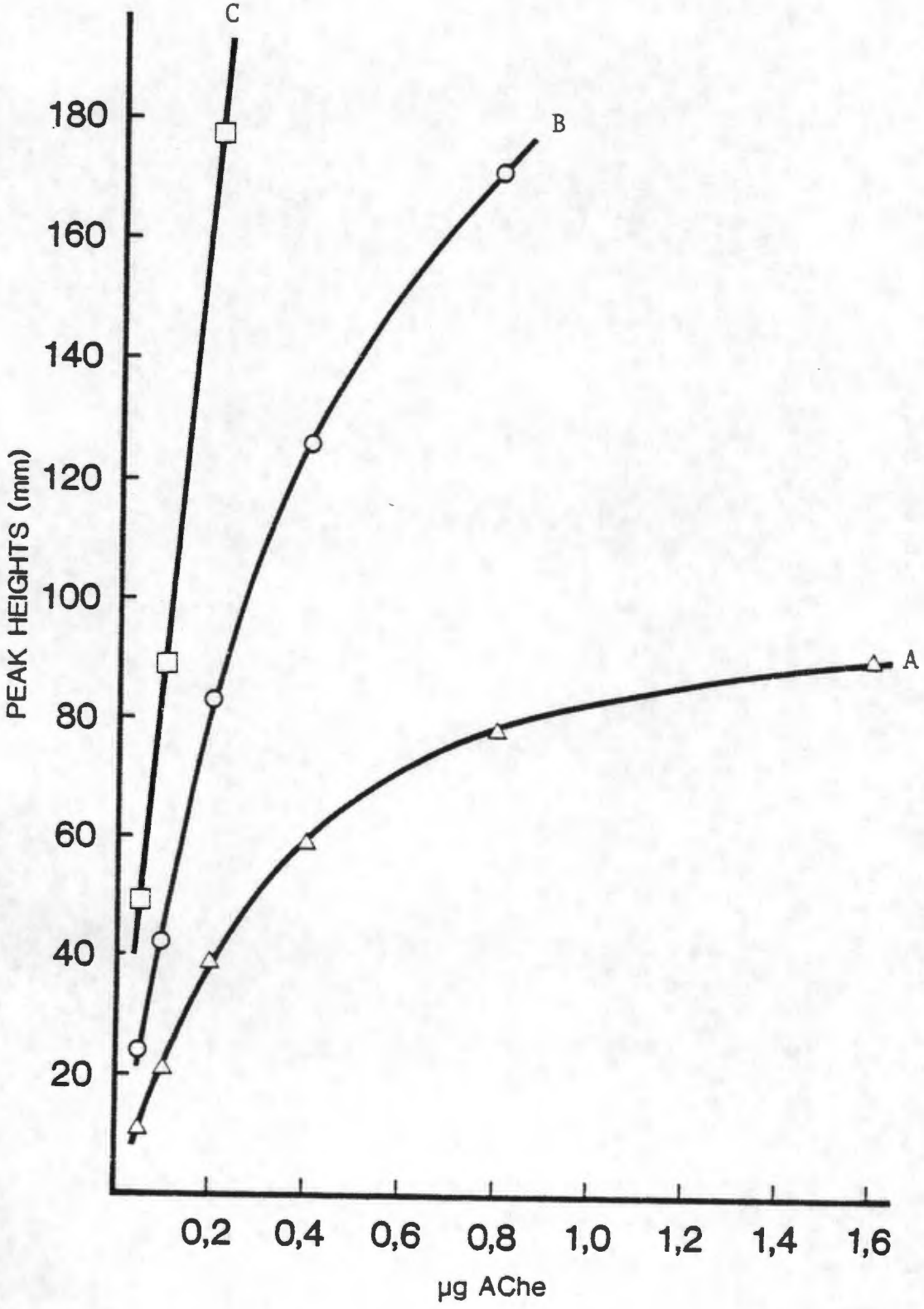
AChE conc.		PEAK HEIGHTS (mm)		
µg/ml	µg/8µl	+ 2V	+ 1V	+ 500mV
12,5	0,05	11	24	49
25	0,1	21	42	89
50	0,2	39	83	177
100	0,4	59	126	*
200	0,8	78	171	*
400	1,6	90	*	*

* Scan off scale.

Fig. 5.11.

RELATIONSHIP BETWEEN PEAK HEIGHTS AND PURE AChE (µg/8µl) MEASURED ON THE ZEISS DENSITOMETER AT

A : + 2V
B : + 1V
C : + 500 mV



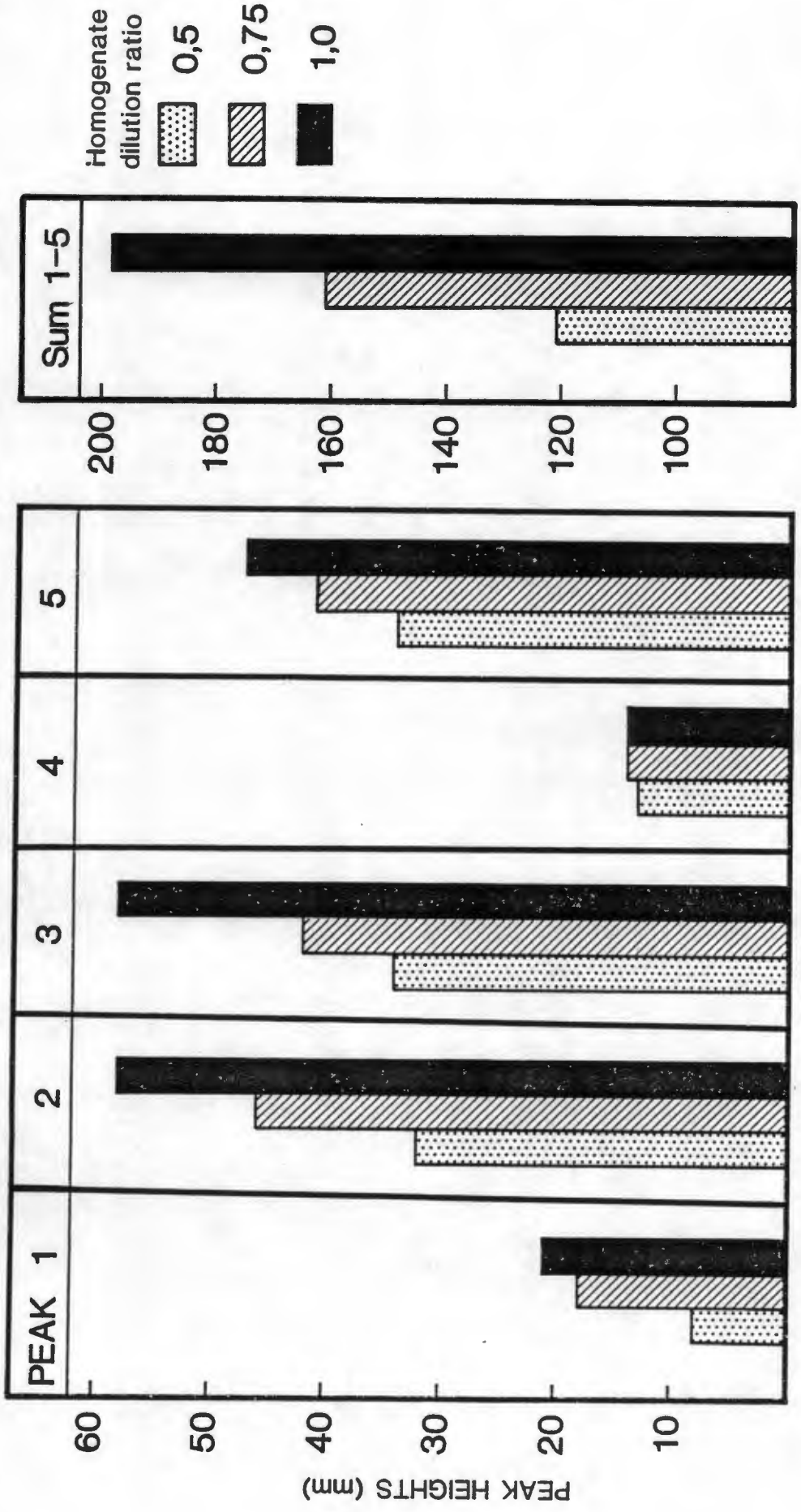
T A B L E 5.2.

RELATIONSHIP BETWEEN FETAL MOUSE BRAIN HOMOGENATE CONCENTRATIONS AND PEAK HEIGHTS MEASURED AT + 500 mV

		MOUSE BRAIN HOMOGENATE		
mg/ml		12mg/ml	18mg/ml	24mg/ml
Dilution ratio		0,5	0,75	1,0
PEAK HEIGHTS (mm)	Peak 1	8	18	21
	(Dilution Ratio)	(0,38)	(0,86)	(1,0)
	2	32	46	58
	(Ratio)	(0,55)	(0,79)	(1,0)
	3	34	42	58
	(Ratio)	(0,59)	(0,72)	(1,0)
	4	13	14	14
	(Ratio)	(0,93)	(1,0)	(1,0)
	5	34	41	47
	(Ratio)	(0,72)	(0,87)	(1,0)
	Sum 1-5	121	161	198
	(Ratio)	(0,61)	(0,81)	(1,0)

Fig. 5.12.

RELATIONSHIP BETWEEN PEAK HEIGHTS AND MOUSE BRAIN HOMOGENATE DILUTIONS



The linear relationship was most apparent at a high sensitivity setting of the densitometer (i.e. + 500 mV full scale deflection as opposed to + 1 or + 2V).

Peak heights are a measure of the stain intensity of the AChE band. Therefore, at low AChE concentrations, stain intensity or dye uptake should be proportional to the actual concentration of AChE. The intensity of staining of fetal mouse brain AChE isoenzyme bands fell within this region of lower AChE concentrations above (see Fig. 5.10). The assumption was therefore made of a linear relationship between peak heights and AChE concentration for fetal mouse brain isoenzymes. The loss of linearity at high concentrations was explicable on the basis of excessive staining i.e. as the staining became denser the illuminating light could no longer penetrate adequately (the problem was apparent on examining the darker bands in Fig. 5.10).

There appeared to be an approximate linear relationship between peak heights and brain homogenate AChE concentration but this was not apparent for all the isoenzyme bands. The peak heights for bands 2 and 3 showed a 1 : 0,79 : 0,55 and 1 : 0,72 : 0,59 ratio respectively, which was comparable with the dilution ratio of 1 : 0,75 : 0,50.

The ratios for the peak heights for bands 1, 4 and 5 were less comparable with the dilution ratios but the sum of the peak heights for all 5 bands had similar ratios to those of the dilutions (see Fig. 5.12). Band 4 tended to be very faint, (see Fig. 5.12) particularly at the lower homogenate concentrations. The accuracy with which the densitometer could measure the intensity of this band against the relatively high background level, was therefore probably reduced quite considerably.

The peak heights for band 5 showed a 1 : 0,87 : 0,72 ratio. Although not a linear relationship there was nevertheless a relationship between peak height and concentration. The lack of a true linear relationship may be explained by the fact that band 5 was a rather poorly resolved band. Similarly, for band 1, although there was not a linear relationship, a relationship between peak height and concentration was apparent. A lack of a true linear relationship in this instance is probably best explained by the higher proportion of interference from other peaks.

From the above results for fetal mouse brain homogenate, it appeared that bands 2 and 3 and the sum of all 5 bands, and probably bands 5 and 1, were likely to give reasonable AChE representations. If there was increased activity of band 4 a similar assumption could probably be made.

5.8. POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE ISOENZYMES OF 18 DAY C3H MOUSE BRAIN ACETYLCHOLINESTERASE : CONCLUSIONS

a) Five distinct zones were apparent.

b) Inhibition studies confirmed that the bands were AChE.

c) The technique was reproducible.

d) There was good separation and resolution of the bands, the best resolved being bands 2, 3 and 4. Bands 2 and 3 had the highest activity.

e) The bands were suitable for densitometric analysis where 5 main peaks were demonstrable and corresponded well to the 5 zones on the gel.

f) The assay gave a reasonable representation of AChE isoenzyme concentration with bands 2 and 3 and the total sum of the peaks, and probably with band 5.

g) The concentrations of the isoenzymes represented by peaks 1 and 4, however were probably difficult to measure since the peaks were short and their amplitude was much confounded by background scatter.

CHAPTER 6

A STUDY OF THE EFFECTS OF IN VIVO MATERNAL ADMINISTRATION OF LARGE DOSES OF VITAMIN A ON THE C3H MOUSE FETUS

6.1. INTRODUCTION

A discussion on vitamin A as a teratogen, with particular reference to its effects on the developing CNS, has been given in section 1.3.

The aims of this study were:

i) To investigate the effects of vitamin A when administered during embryonic development, including an important period of CNS development, on gross fetal parameters in C3H mice including embryolethality, morphological abnormalities, fetal weight and brain weight;

ii) to compare these with the effects on brain AChE and its isoenzymes, and with brain choline acetyltransferase;

iii) to evaluate AChE as a biochemical marker of teratogenic injury;

iv) to elucidate the teratogenic mechanisms of vitamin A with particular reference to the CNS.

6.2. MATERIALS AND METHODS

6.2.1. Experimental mice

Timed matings were performed with young healthy virgin female C3H mice aged 8 - 12 weeks and weighing 20 - 23g, as described in section 2.4, and kept under standard experimental conditions (section 2.3). Over-

night matings were performed for most experiments in the pilot study, but for the definitive study 2h matings were performed. For overnight matings the following day was designated day ½ of pregnancy. For 2h matings the day of copulation was designated day 0 p.c. Pregnant females were randomly divided into test and control groups.

6.2.2. Drug administration

Vitamin A was administered by gastric intubation using a 1ml Tuberculin syringe with a special blunt ended needle on one or more days from day 7 - 13 p.c. The doses used were 1 000 - 50 000 IU vitamin A per mouse. An equal volume of arachis oil was administered to controls.

6.2.3. Records of maternal and fetal parameters and statistical analysis

Maternal parameters

The weight on the day of mating, day of dosing and the day of sacrifice and the physical state of each mouse after dosing and before sacrifice were recorded.

Fetal parameters

Resorptions, intra-uterine deaths (IUD's) and gross morphological abnormalities were recorded in the preliminary study. These parameters were also recorded in the definitive study as well as fetal weight, brain weight, brain AChE activity, and in some experiments AChE isoenzymes and choline acetyltransferase activity.

Statistical analysis

The incidence of resorptions, intra-uterine deaths and morphological abnormalities was compared with controls using the Chi-square two-tailed test. Fetal weights, brain weights and brain AChE values were compared with controls using the non-parametric Mann-Whitney U two-tailed test.

Medians and 95% confidence limits were calculated from the Walsh averages, after Tukey (Steinijans and Diletti, 1983).

6.3. PRELIMINARY EXPERIMENTS

Aims

As discussed in section 1.2. the toxic expression of a teratogenic agent may be influenced by the genotype of the conceptus, the developmental stage at the time of exposure, and the dose used. Guided by the experimental literature, as presented in section 1.2, the susceptibility of the C3H inbred strain to vitamin A under the experimental conditions used was determined. The second aim of the study was to determine a suitable dosage range and time of administration of vitamin A for subsequent experiments with brain AChE with the following in mind: a) to find a suitable "sub-teratogenic" dose using standard criteria of resorption rate, fetal death and morphological abnormalities; and b) to find a suitable teratogenic dose (without producing too high an incidence of resorption or IUD's which would preclude AChE analysis, and without producing an excessive incidence of gross CNS abnormalities which would be expected to be associated with enzyme changes). Further aims of the study were (i) to determine that the doses used were less than the minimal toxic dose for the mothers and (ii) to determine whether vitamin A per se affects AChE.

The dose range of vitamin A chosen was 1 000 - 50 000 IU per mouse which from the literature was considered likely to give a spectrum of effects from non-teratogenic to embryolethal. The time of administration chosen was from day 8 to 10 p.c., generally a single administration, since this falls within an important period of CNS development (see section 1.2.) Dams were sacrificed and fetal parameters recorded on days 18 and 19 p.c. Table 6.1. lists the preliminary experiments performed.

TABLE 6.1.

VITAMIN A : PRELIMINARY EXPERIMENTS

Dose of Vitamin A	Day of administration (post conception)
50 000	9½
15 000	8½ and 9½
15 000	9½
10 000	9½
10 000	10
5 000	9½
1 000	9½

6.3.1. Vitamin A, 50 000 IU per mouse, administered on day 9½ p.c.

Four C3H female mice were used in the test group and three in the control group.

Results are shown in Tables 6.2T and 6.2C (pp. 327 and 328).

Note Tables of original data are presented at the end of the thesis Postscript "T" attached to a Table number designates test group and "C" the control group.

By the day of sacrifice the treated dams were noted to have become scrawny with hair loss suggesting a maternal toxic effect. All dams were pregnant but there was a 100% resorption rate with no live fetuses. In the control group there were 21 normal fetuses with one resorption and one intra-uterine death.

6.3.2. Vitamin A, 15 000 IU per mouse, administered on days 8½ and 9½ p.c.

Five mice were used in the test group and three in the control group.

Results are shown in Tables 6.3T and 6.3C (pp. 329 and 330).

Although the dams appeared to be healthy at the time of sacrifice there were 27 resorptions in the test group (96,4%) and only one live abnormal fetus with exophthalmos, a hypoplastic face and short tail. In the control group there were 17 fetuses, 16 of which were normal and one had exomphalos.

6.3.3. Vitamin A, 15 000 IU per mouse, administered on day 9½ p.c.

Five mice were used in the test group and four in the control group.

Results are shown in Tables 6.4T and 6.4C (pp. 331-334).

In the test group there were 21 fetuses. There was a 25,8% resorption rate compared with 3,7% in controls ($p<0,001$, Chi-square test), a 6,4% incidence of intra-uterine deaths which was not significant, and a 66,6% incidence of morphological abnormalities compared with 4,2% in controls ($p<0,001$). Abnormalities noted were exopthalmos, abnormal shaped head and absent or crooked tail. One fetus had a myelomeningocele and can best be described as a monster. In the control group there were two macerated IUD's, and one of the 24 live fetuses was abnormal with exomphalos.

6.3.4. Vitamin A, 10 000 IU per mouse, administered on day 9½ p.c.

Nine mice were used in the test group and eight in the control group (two separate experiments).

Results are shown in Tables 6.5T, 6.5C, 6.6T and 6.6C (pp. 335-339).

In the test group there was a 13,4% resorption rate, a 2,4% incidence of intra-uterine deaths and 40,8% of fetuses had gross morphological abnormalities. The most common abnormality in 41% of the fetuses was proptosed eyes with open eyelids (exophthalmos), which was associated with a characteristic abnormal shape of the head (see Figs. 6.1. and 6.2. and compare with a normal fetus, Fig. 6.3). Two fetuses were exencephalic. In the control groups there were 37 fetuses, 36 of which were normal. One had exomphalos (Fig. 6.4). There were 7 resorptions (15,2%) and two intra-uterine deaths.

6.3.5. Vitamin A, 10 000 IU per mouse, administered on day 10 p.c. (Two hour mating)

Two separate experiments were performed, each with three mice in the test group and three in the control group.

Results are shown in Tables 6.7T, 6.7C, 6.8T and 6.8C (pp. 340-343).

In the test group 64,7% of fetuses had gross morphological abnormalities. The most common abnormalities were defects of the tail which were either absent (Fig. 6.5), short, broad or curled. There were 12,2% resorptions and 4,9% intra-uterine deaths. In the control group there were 43 fetuses, all of which were normal, three resorptions and three intra-uterine deaths.

6.3.6. Vitamin A, 5 000 IU per mouse, administered on day 9½ p.c.

Four mice were used in the test group and four in the control group.

Fig. 6.1.

18-DAY FETUS WITH EXOPHTHALMOS AND ABSENT TAIL

Mother treated with 10 000 IU vitamin A on day 9½ post conception.

Magnification x 3.

Note:

- exophthalmos
- abnormal shaped head
- shortened tail
- shortened mandible
- absent tail

Fig. 6.2.

18-DAY FETUS WITH EXOPHTHALMOS

Mother treated with 10 000 IU vitamin A on day 9½ post conception.
Magnification x 22.

Note:

- protruding eyes (exophthalmos)
- abnormal shaped head
- (artifacts are air-bubbles)

Fig. 6.3.

NORMAL 18-DAY FETUS

Magnification x 5.

Note:

- shape of head
- closed eye
- normal tail

Fig. 6 .4.

18-DAY FETUS WITH EXOMPHALOS

Mother untreated (spontaneous abnormality). Magnification x 1,8.

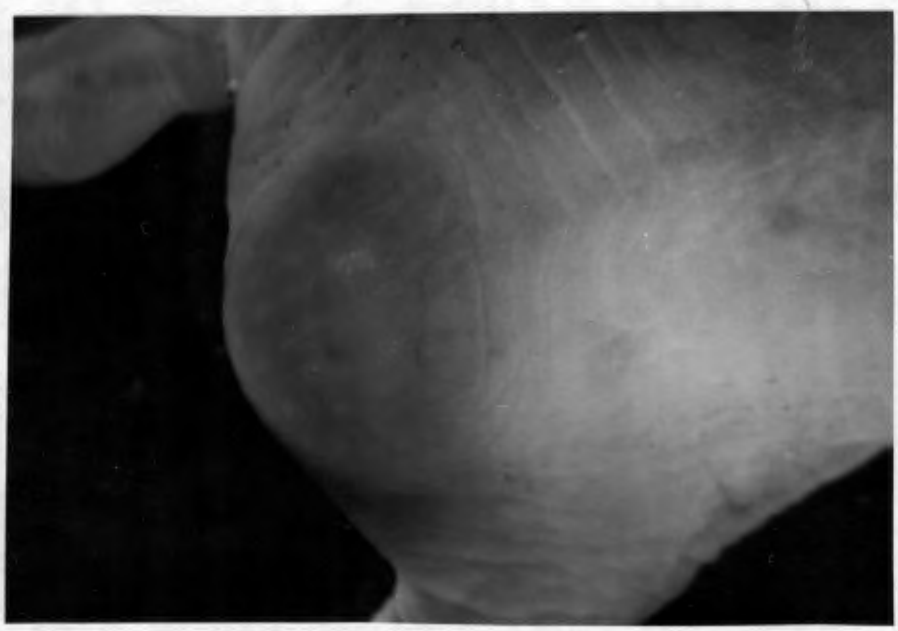
Note:

- herniation of intestines
- short tail

Fig. 6.5.

18-DAY FETUS WITH ABSENT TAIL

(MAGNIFIED VIEW OF FETAL HIND-QUARTERS)



Mother treated with 10 000 IU vitamin A on day 10 post conception.

Magnification x 20.

Note:

- absent tail with probable underlying meningocoele.

Results are shown in Tables 6.9T and 6.9C (pp. 344-346).

In the test group there were 28 fetuses. Twenty-five were normal, two had slightly prominent eyes, possibly suggesting mild exophthalmos, and one fetus had exomphalos. There was one resorption and no intra-uterine deaths. In the control group there were 27 fetuses, all normal (two pairs of twins were small), one intra-uterine death and one resorption.

6.3.7. Vitamin A, 1 000 IU per mouse, administered on day 9½ p.c.

Three mice were used in the test group and three in the control group.

Results are shown in Tables 6.10T and 6.10C (pp. 347-348).

In the test group there were 19 fetuses, all normal, one resorption and one intra-uterine death. In the control group there were 18 normal fetuses, one resorption and one intra-uterine death.

6.3.8. Study to determine whether doses of vitamin A used were below the minimal toxic dose level (M.T.D.) for the adult C3H females used.

If an agent is toxic to the mother this maternal toxicity can obviously confound the fetal results. It was therefore important to know whether the doses of vitamin A used were below the M.T.D. for the C3H female mice used (see Section 2.9). Non-pregnant female C3H mice weighing 19 - 23g were removed from stock cages of 10 - 15 mice per cage and placed 3 per cage 10 days prior to dosing to enable adjustment to these conditions. Ten thousand IU vitamin A was administered to 15 animals on day 0 by gastric intubation. Animals were numbered by means of ear-punching and kept 3 per cage under standard conditions. They were given food and water ad libitum and

observed for signs of toxicity. The mice were reweighed 9 days after dosing (a commonly used time of sacrifice for pregnant females).

Results

No signs of toxicity or deaths were noted during the 9 days after dosing. Table 6.11. gives the mouse weights on the day of dosing (day 0) and on day 9 and the change in weight. Nine of the mice gained weight, in 3 the weight remained the same and with 3 there was a slight reduction of 0,4 - 0,5gms.

These results suggest that a dose of 10 000 IU vitamin A did not have a significant toxic effect on adult C3H female mice. (A dose of 10 000 IU was the maximum dose used in all but 2 subsequent experiments in this study).

6.3.9. Experiment to determine whether vitamin A, independent of its teratogenic effects, alters brain AChE activity

Vitamin A, 10 000 IU, which had been shown to be teratogenic when administered on day 9 or 10 p.c., was administered on day 17 p.c. i.e. during the late period of fetogenesis when susceptibility to teratogenic effects is unlikely. The mice were sacrificed on day 19 p.c.

Results are shown in Table 6.12T and 6.12C (pp. 349-350). A summary of results is given in Table 6.13.

There were no gross abnormalities in the treated group and no resorptions in either the test or control groups. There was no significant difference in brain AChE activity between tests and controls, nor in fetal nor brain weight. The absence of any difference in AChE between the group treated with vitamin A and controls suggests that vitamin A, independent of its embyrotoxic effects, does not alter fetal brain AChE activity.

T A B L E 6.11.

MOUSE WEIGHTS ON DAY OF ADMINISTRATION OF VITAMIN A (DAY O)

AND 9 DAYS LATER (DAY 9)

WEIGHT IN GRAMS		
Day O	Day 9	Weight Change
21,5	21,5	O
20,0	20,2	+ 0,2
19,5	19,8	+ 0,3
21,0	20,6	- 0,4
19,5	20,0	+ 0,5
22,5	22,5	O
20,0	22,0	+ 2,0
20,0	19,5	- 0,5
20,5	21,0	+ 0,5
22,0	21,5	- 0,5
19,5	20,0	+ 0,5
19,5	19,5	O
21,5	22,0	+ 0,5
19,5	20,0	+ 0,5
22,0	22,5	+ 0,5

T A B L E 6.13.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A

ADMINISTERED ON DAY 17 p.c.

	TEST	CONTROL	p
Total Conceptions	19	18	-
Resorptions (%)	9,5	5,26	N.S.
Intra-uterine Deaths (%)	0	5,26	N.S.
Abnormalities (%)	5,26	0	N.S.
Median Fetal Weight (g)	1,28 (1,25 - 1,32)	1,285 (1,25 - 1,32)	N.S.
Median Brain Weight (mg)	84,88 (83,2 - 85,9)	83,85 (82,6 - 84,5)	N.S.
Median AChE Activity nmol/min/mg	3,36 (3,25 - 3,43)	3,265 (3,18 - 3,34)	N.S.

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

6.3.10. The basis for exophthalmos

Exophthalmos was a striking and common abnormality induced by vitamin A. There is controversy in the literature regarding the basis of the anomaly and this was briefly examined.

Materials and methods

After maternal administration of 10 000 IU vitamin A on day 9 p.c., fetuses with exophthalmos, and normal control fetuses, were fixed in 10% neutral buffered formalin on day 18 p.c. Heads were processed for paraffin sections and cut at 7µm thickness before staining with haematoxylin and eosin. Frontal sections taken through the centre of the eyeball of treated fetuses were compared. Measurements were obtained with the aid of a MOP - videoplan Image Analysis System (Kontron, Munich, West Germany).

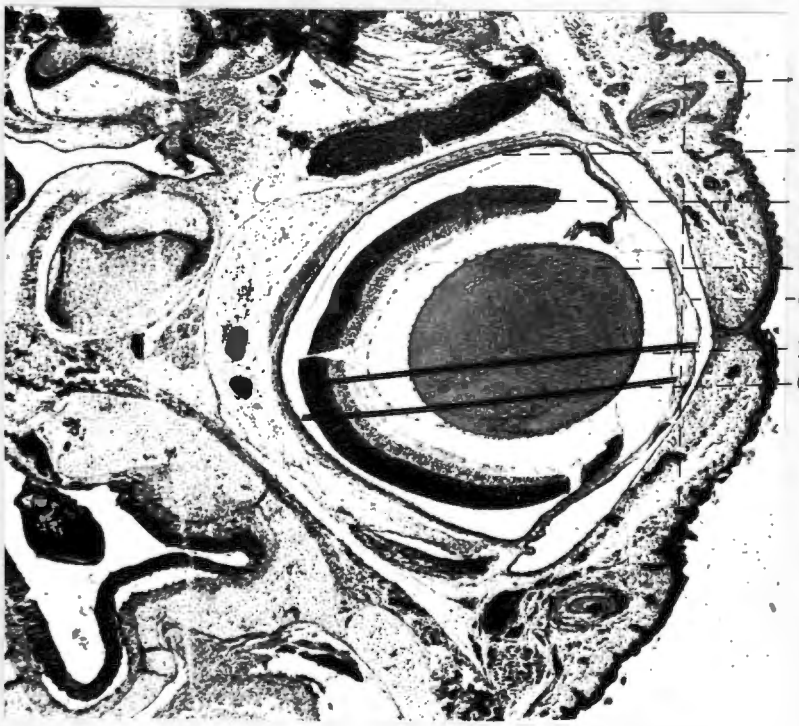
Results

Fig. 6.6 and 6.7 are photographs of frontal sections through the centre of the orbit and eyeball of a normal fetus and of a fetus with exophthalmos, respectively. Although the exophthalmic eyeball appears normal it was shorter than that of the normal eye (43,8mm versus 54,2mm, respectively), and the exophthalmic eye is obviously proptosed due to the shallow orbit (24,7mm versus 55,8mm of the control).

Discussion

The above findings are in agreement with those of Kalter and Warkany (1961) who suggested that the basis for exophthalmos induced by vitamin A in the fetal mouse was reduction in the size of the orbit, probably due to defective development of the facial skeleton. The eye itself was normal. Geelan (1979) also noted that the ocular bulb protruded due to underdevelopment of the orbit. The foregoing is at variance with the findings of Padmanabhang and Singh (1981) who intimated that exophthalmos in rat fetuses was due to an increased amount of primary vitreous and a larger retroretinal space.

Fig. 6.6.



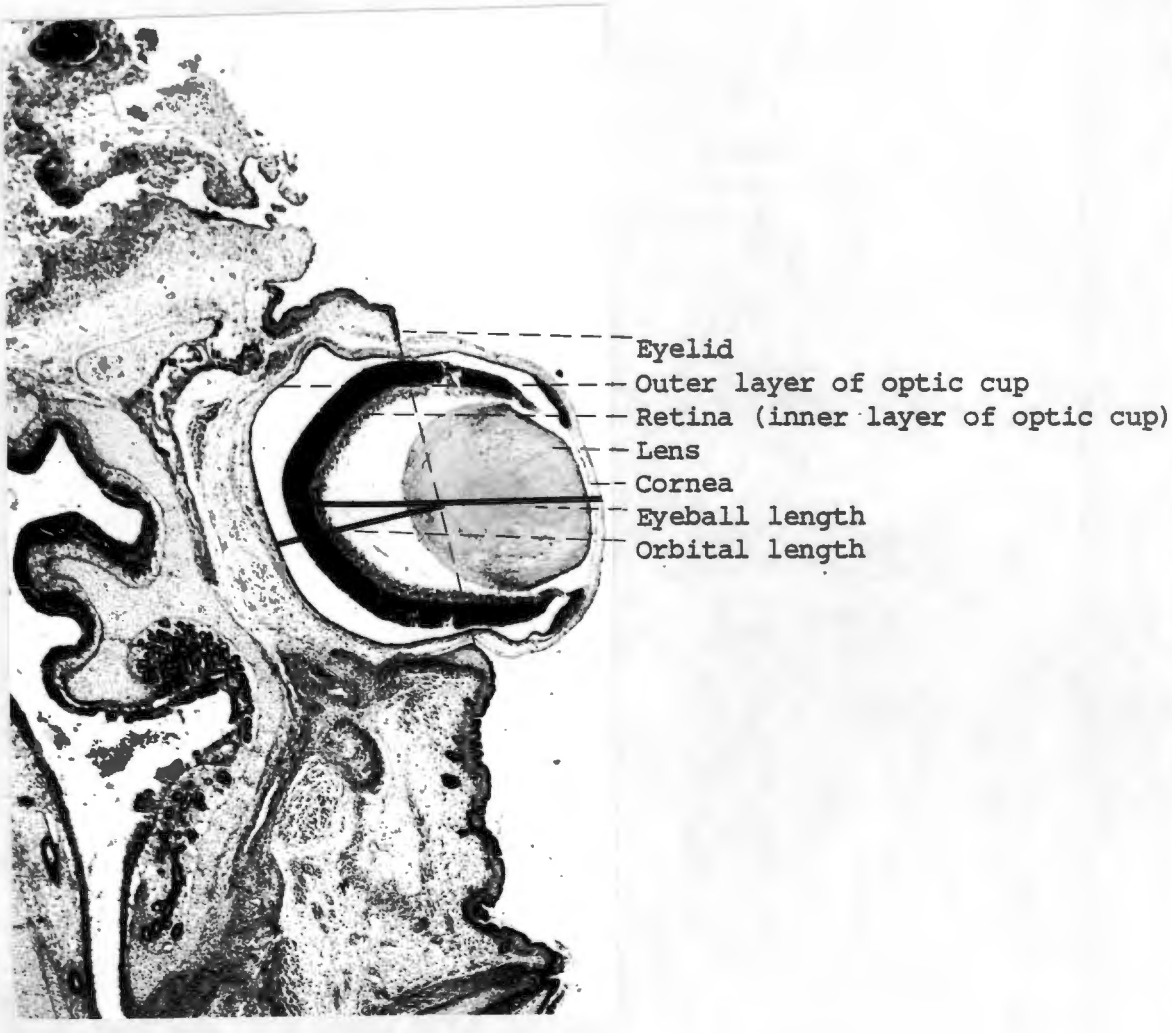
Magnification x 40

Frontal section through the centre of the eyeball of a normal 18-day fetus

Note:

The closed eyelids
The normal ocular bulb
The normal orbit

Fig. 6.7.



Magnification x 40

Frontal section through the centre of the eyeball in an 18-day fetus with exophthalmos after maternal administration of 10 000 IU vitamin A on day 9 p.c.

Note: The open eyelid
The proptosed eye
The grossly normal ocular bulb
The shallow orbit

6.3.11. Discussion and Conclusions of Preliminary Experiments

Table 6.14 gives a summary of the effects of vitamin A on fetal outcome and the level of significance. Table 6.15 gives the details of the gross abnormalities produced by vitamin A.

A single dose of 10 000 IU vitamin A administered on day 9½ or on day 10 p.c. was obviously teratogenic with a 40,8% and 64,7% incidence of abnormalities respectively, compared with 2,7% and 0% in controls ($p = 0,001$, Chi-square test). The most common abnormality with dosage on day 9½ p.c. was exophthalmos which was present in 41% of fetuses. Other abnormalities were exencephaly (4,1%) and absent tail (8,2%). Dosage on day 10 resulted mainly in tail abnormalities, which were present in 50% of fetuses. Exophthalmos was present in 5,9% and nasal blebs in 8,8% ($\pm$ 1mm cystic blebs, one on either side of the midline over the maxillo-frontal region. These were not observed as spontaneous abnormalities and were a feature of vitamin A embryotoxicity).

With a dose of vitamin A of 1 000 IU on day 9½ p.c. there were no morphological abnormalities, one resorption and one intra-uterine death which was no different to controls ($p = 0,5$, Chi-square test). Although a small sample the results suggested that 1 000 IU was not a teratogenic dose.

With a dose of 5 000 IU on day 9½ p.c. there was no significant difference in the incidence of resorptions or intra-uterine deaths but the presence of slightly prominent eyes in two of the fetuses, perhaps indicating mild exophthalmos, may have suggested a teratogenic effect in these two fetuses.

A dramatic increase in resorption rates was apparent with increasing doses of vitamin A above 10 000 IU, the incidence rising from 25,8%

TABLE NO. 6.14. SUMMARY OF THE EFFECTS OF VITAMIN A ON FETAL OUTCOME : PILOT STUDY

DOSE I.U.	50000		15000 x 2		15000		10000		10000		5000		1000	
DAY OF DOSING	9½		8½ + 9½		9½		9½		10		9½		9½	
	T	C	T	C	T	C	T	C	T	C	T	C	T	C
TOTAL CONCEPTIONS	25	23	28	18	31	27	82	46	41	43	28	29	21	20
% RESORBED	100	4,3	96,4	5,5	25,8	3,7	13,4	15,2	12,2	7,0	0	3,4	4,7	5,0
P	< 0,001		< 0,001		< 0,001		NS		0,001		NS		NS	
% I.U.D.	-	4,3	-	0	6,4	7,4	2,4	4,3	4,9	7,0	0	3,4	4,7	5,0
P	-		-		NS		NS		NS		NS		NS	
% MORPHOLOGICAL ABNORMALITIES	-	0	-	5,9	66,6	4,2	40,8	2,7	64,7	0	10,7	0	0	0
P	-		-		< 0,001		0,001		< 0,001		0,02		-	

T = test group
C = control group
P = level of significance (Chi-square test)

TABLE NO. 6.15.

PRELIMINARY EXPERIMENTS : GROSS MORPHOLOGICAL ABNORMALITIES PRODUCED BY VITAMIN A

DOSE IU	10 000		15 000		10 000	
Day of Dosing	9½		9½		10	
Total no. of Fetuses	49		21		34	
	No.	%	No.	%	No.	%
Exencephaly	2	4,1	0	0	0	0
Exophthalmos/ abnormal shaped head	20	41	11	52,4	2 (3 pro-minent eyes)	5,9
Exomphalos	2	4,1	0	0	2	5,9
Tail abnormalities	4	8,2	0	0	17	50
Nasal blebs	0	0	0	0	3	8,8%

with 15 000 IU on day 9½ p.c. to 96,4% with 15 000 on days 8½ and 9½ p.c. and 100% with 50 000 IU on day 9½. For the purposes of studying brain enzymes these latter two doses were therefore unsuitable. The resorption rates of 13,4% and 12,2% with 10 000 IU were higher than those with 1 000 and 5 000 IU (4,7% and 0%) and are compatible with an embryolethal effect, although the wide variation in resorption rates observed in controls (3,4 - 15,2%) precludes any meaningful conclusion.

There was no significant difference in the incidence of intra-uterine deaths compared with controls with any of the doses used (the 96,4% and 100% resorption rate with 30 000 and 50 000 IU precluded the possibility of IUD's).

With doses of vitamin A up to 10 000 administered on day 9½ or 10 p.c., gross morphological abnormalities were a useful indicator of a teratogenic effect. Dysmorphogenic effects (Table 6.15) differed between dosing on day 9½ (exophthalmos in 41% of fetuses, exencephaly in 4,1% and tail abnormalities in 8,2%) and day 10 (tail abnormalities in 50% of fetuses, exophthalmos in 5,9% and nasal blebs in 8,8%). This is consistent with the different developmental stages at the time of exposure.

The dosage range of vitamin A selected for further experiments was 1 000 - 10 000 IU, which appeared to be below the M.T.D. for adult C3H mice. One thousand IU on day 9½ p.c. appeared to be non-teratogenic. This was considered to be a useful dose for determining whether a "sub-teratogenic"dose for gross fetal abnormalities might still be associated with a biochemical abnormality, such as a change in AChE. Five thousand IU on day 9½ p.c. was possibly teratogenic, whereas merely doubling the dose to 10 000 IU on day 9½ or 10 p.c. was obviously teratogenic. Ten thousand IU administered on day 9½ or 10 p.c. was therefore considered a suitable teratogenic dose.

It was apparent that dosing on a single day i.e. day 9 or 10 with vitamin A was adequate to produce teratogenic effects. However, for the definitive study dosing was also performed on more than one day, to enable longer fetal exposure to the teratogen, and also on various days of gestation to compare the effects during different developmental stages.

Although fetal weight is another useful parameter for monitoring embryotoxic effects fetuses were not weighed in the above experiments. The incidence of morphological abnormalities, resorptions and intrauterine deaths was adequate for the purposes of this preliminary study. However, for further definitive studies all fetal and brain weights were recorded.

The only gross macroscopically apparent spontaneous abnormality among controls was exomphalos (herniation of the intestines through a defect in the anterior abdominal well), present in 2 of all fetuses. Exomphalos was also present in 4 of all test fetuses.

6.4. RESULTS OF DEFINITIVE VITAMIN A STUDIES

Tables of original data are presented at the end of the thesis.
Summary Tables are given with the text.
Table 6.16. shows the doses used, the day(s) of administration and the day of sacrifice and the specific assay performed.

6.4.1. Vitamin A, 1 000 IU per mouse, administered on day 9 p.c. (sacrifice day 18)

See Tables 6.17T and 6.17C for original data (pp. 351-354).
Summary of results in Table 6.18.

TABLE 6.16.

VITAMIN A STUDY : SUMMARY OF EXPERIMENTS PERFORMED SHOWING DOSE OF VITAMIN A, DAY OF ADMINISTRATION AND SACRIFICE, AND THE SPECIFIC ASSAY PERFORMED

Dose of Vitamin A (I.U.)	Day(s) of Administration	Day of Sacrifice	Assay
1 000	9	18	Whole brain AChE
5 000	9	18	"
5 000	10	14	"
5 000	10	12	"
10 000	10	18	"
10 000	10	19	"
10 000	13	18	"
10 000	9	19	"
10 000	9	12	"
10 000	9	18	Cerebellum AChE
10 000	9	18	"
10 000	9	18	Cortex AChE
5 000	8,9	19	*
7 500	8	19	*
2 500	7,8,9	18	*
2 500	7,8	19	*
3 000	8,9	18	Whole brain AChE
10 000	10	18	Total protein content
10 000	10	18	Whole brain ChAT
15 000	10	18	"
10 000	10	18	Isoenzymes

* Embryolethality precluded AChE assay.

T A B L E 6.18.

SUMMARY OF RESULTS OF 1 000 IU VITAMIN A ADMINISTERED ON DAY 9 p.c.

	TEST	CONTROL	p
Total Conceptions	31	32	-
Resorptions (%)	3,2	3,1	NS
Intra-uterine Deaths (%)	10,0	3,1	NS
Abnormalities (%)	0	0	NS
Median Fetal Weight (g)	0,866 (0,83 - 0,92)	0,85 (0,81 - 0,87)	NS
Median Brain Weight (mg)	63,6 (61,5 - 65,7)	62,35 (61,3 - 63,4)	NS
Median AChE Activity nmol/min/mg	2,25 (2,15 - 2,45)	2,32 (2,28 - 2,37)	NS

Figures in brackets are the 95% confidence range
For statistical tests used see Section 6.2.3.

Note

There were no signs of maternal toxicity in this or any of the subsequent experiments with vitamin A in this chapter. Weight gain appeared similar after dosing to that of controls but was not statistically compared because of the small numbers in each group.

There were no gross morphological abnormalities. There was one resorption in the test group and one in the control group, and there were three intra-uterine deaths in the test group and one in the control group. There was no significant difference in fetal weight, brain weight or brain AChE compared with controls.

Discussion

There was no significant difference in any of the above six parameters compared with controls. As was suggested by the pilot study a single dose of 1 000 IU vitamin A administered on day 9 p.c. did not appear to be teratogenic.

6.4.2. Vitamin A, 5 000 IU per mouse, administered on day 9 p.c. (sacrifice day 18)

See Tables 6.19T and 6.19C for original data (pp. 355-357).

Summary of results in Table 6.20.

Two of the fetuses in the test group had exophthalmos and one was a runt. In the control group two of the fetuses were runts and had a slight abnormality in the form of a haemorrhagic bullus over the dorsal midline of the nose. There were five resorptions in the test group and three in the control group. There were three intra-uterine deaths in the test group and none in the control group. No significant difference was apparent in fetal weight, brain weight or brain AChE compared with controls.

T A B L E 6.20.

SUMMARY OF RESULTS OF 5 000 IU VITAMIN A

ADMINISTERED ON DAY 9 p.c.

	TEST	CONTROL	p
Total Conceptions	30	23	-
Resorptions (%)	16,7	13,0	NS
Intra-uterine Deaths (%)	10,0	0	0,1
Abnormalities (%)	9,1	0	NS
Median Fetal Weight (g)	0,97 (0,92 - 1,03)	1,012 (0,96 - 1,06)	NS
Median Brain Weight (mg)	71,0 (67,7 - 72,3)	70,0 (66,5 - 72,3)	NS
Median AChE Activity nmol/min/mg	2,69 (2,60 - 2,79)	2,75 (2,68 - 2,81)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

Discussion

From the pilot study and subsequent studies exophthalmos was a characteristic abnormality induced by vitamin A when administered on day 9 p.c. and was never observed in control animals. A gross teratogenic effect was therefore apparent in two of the treated fetuses. However, there was no significant difference in brain AChE activity nor fetal or brain weight of treated animals compared with controls. The three intra-uterine deaths in the test group compared with none in the control group ($p = 0,1$) could have been due to an embryotoxic effect of vitamin A, but as many as three IUD's have been noted in the same sized sample of untreated animals in other experiments and is therefore of questionable significance. Similarly, the five resorptions in the test group compared with three in the control group could be explained on either basis. The presence of gross abnormalities in only two fetuses in the absence of any other significant effects could have indicated that this dose of vitamin A exerted a teratogenic effect only on a small number of highly susceptible fetuses with no effect on the majority of fetuses. Alternatively, if the dose response curve for biochemical changes was close to that for malformations a low teratogenic response with regard to malformations would be expected to be associated with a low incidence of biochemical changes. Further larger studies to increase the chance of achieving statistical significance were not performed.

Another explanation for the lack of a significant change in AChE with lower doses of vitamin A i.e. 1 000 and 5 000 IU is that repair of any teratogenic damage to the CNS could have been complete by the time of sacrifice (day 18 p.c.) (see section 1.1). Sacrifice was therefore performed earlier (day 14) in the next experiment.

6.4.3. Vitamin A, 5 000 IU per mouse, administered on day 10 p.c. (sacrifice day 14)

Because of the technical difficulty of removing brains from such minute friable 14-day fetuses and because most of the head is occupied by the developing brain at this age, whole heads were assayed for AChE activity. Decapitation was performed by a horizontal incision in line with the bottom of the lower jaw.

See Tables 6.21T and 6.21C for original data (pp. 358-359).

A summary of results is given in Table 6.22.

There were two resorptions in the test group and two in the controls. No gross morphological abnormalities were apparent but because of the small size of the fetuses minor abnormalities might have been missed. Compared with controls, no difference in fetal weight, head weight or AChE activity was established.

Discussion

As with sacrifice on day 19 there was no significant difference in AChE compared with controls. It would therefore appear unlikely that repair accounted for the normal AChE activity, unless repair was complete by day 14. Of note was the lower AChE activity in 14-day fetuses compared with 18 or 19 day fetuses (approximately 1/3). Although this is explicable on the basis of the younger age and more immature nervous system (see section 3.3) using the whole head would have contributed some dilutional effect. Unfortunately, this lower enzyme activity made demonstration of subtle changes in AChE levels more difficult.

Because of the possibility that repair might have been complete by day 14, in the next experiment sacrifice was performed earlier on day 12.

T A B L E 6.22.

SUMMARY OF RESULTS OF 5 000 IU VITAMIN A ADMINISTERED ON DAY 10 p.c. (SACRIFICED DAY 14 p.c.)

	TEST	CONTROL	p
Total Conceptions	24	24	-
Resorptions (%)	8,3	8,3	NS
Intra-uterine Deaths (%)	0	0	-
Abnormalities (%)	0	0	-
Median Fetal Weight (mg)	182,5 (174,0 - 191,01)	187,8 (181,0 - 194,0)	NS
Median Head Weight (mg)	66,50 (66,95 - 70,85)	68,55 (65,45 - 70,90)	NS
Median AChE Activity nmol/min/mg	1,017 (0,96 - 1,06)	1,033 (1,0 - 1,06)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

6.4.4. Vitamin A, 5 000 IU per mouse, administered on day 10 p.c. (sacrifice day 12)

See Tables 6.23T and 6.23C for original data (pp. 360-361).

A summary of results is given in Table 6.24.

There were no resorptions in the test group and two in the controls. No gross morphological abnormalities were apparent and there were no IUD's. The fetal weight of 72,1mg was higher for test animals than for controls (64,4mg, $p<0,05$), although the difference in head weights (22,8 and 19,4mg respectively) was not significant. There was no proven difference in AChE activities between test and controls.

Discussion

The higher fetal weight is not readily explicable. (It could have occurred by chance, or possibly by some transient phenomenon such as mild fetal oedema induced by vitamin A. No difference in fetal weights was apparent on day 14 in the previous experiment).

Examination of fetuses 48h after dosing reduced the possibility that repair of any CNS damage might have accounted for an absence of altered AChE activity. However, as already mentioned, using the whole head, and with the lower enzyme activity of the younger embryos, small changes might have been missed.

6.4.5. Vitamin A, 10 000 IU per mouse, administered on day 10 p.c. (sacrifice day 18 and 19)

At this stage I decided to proceed with experiments using an obviously teratogenic dose i.e. 10 000 IU vitamin A. Two consecutive experiments were performed.

See Tables 6.25 and 6.26 for original data (pp. 362-368).

A summary of results is given in Tables 6.27 and 6.28.

T A B L E 6.24.

SUMMARY OF RESULTS OF 5 000 IU VITAMIN A ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 12 p.c.)

	TEST	CONTROL	p
Total Conceptions	20	20	-
Resorptions (%)	0	9,1	NS
Intra-uterine Deaths (%)	0	0	-
Abnormalities (%)	0	0	-
Median Fetal Weight (mg)	72,1 (67,9 - 75,0)	64,4 (57,2 - 70,1)	<0,05
Median Head Weight (mg)	22,8 (20,4 - 24,8)	19,4 (17,1 - 21,6)	NS
Median AChE Activity nmol/min/mg	0,75 (0,72 - 0,80)	0,78 (0,75 - 0,85)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

TABLE 6.27.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 10 p.c. (FIRST EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	32	29	-
Resorptions (%)	9,4	6,9	NS
Intra-uterine Deaths (%)	9,4	10,3	NS
Abnormalities (%)	42,3	0	<0,01
Median Fetal Weight (g)	1,025 (0,975 - 1,061)	1,004 (0,98 - 1,035)	NS
Median Brain Weight (mg)	72,7 (70,2 - 74,9)	68,2 (67,0 - 69,4)	0,001
Median AChE Activity nmol/min/mg	3,155 (3,02 - 3,25)	2,995 (2,93 - 3,05)	0,005

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

TABLE 6.28.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 10 p.c. (SECOND EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	27	23	-
Resorptions (%)	11,1	8,7	NS
Intra-uterine Deaths (%)	7,4	0	NS
Abnormalities (%)	54,5	0	<0,01
Median Fetal Weight (g)	1,227 (1,168 - 1,27)	1,258 (1,16 - 1,32)	NS
Median Brain Weight (mg)	86,65 (84,5 - 88,4)	84,6 (80,2 - 87,1)	NS
Median AChE Activity nmol/min/mg	3,55 (3,40 - 3,61)	3,185 (3,04 - 3,34)	<0,0005

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

Although there was no difference in the rate of resorptions or intra-uterine deaths, the incidence of gross abnormalities in the test groups, 42,3% in the first experiment and 54,5% in the second experiment, was higher than controls($p<0,01$). The most common gross abnormality was a defect of the tail, (50%) which was either absent, short, broad or curled. Two fetuses had exophthalmos (4,2%), 9 (18,8%) had nasal blebs (1mm cystic blebs on either side of the midline over the maxilla) and 10 fetuses (20%) had shortened forelimbs.

In the first experiment the median fetal weight for treated fetuses was higher (1 025mg) than controls (1 004mg), although this was not significant. In the second experiment the median fetal weight was lower for test animals (1 227mg) than controls (1 258mg), but this was also not significant. No conclusion can therefore be drawn.

The median brain weight of 72,7mg in the treated group in the first experiment was significantly higher than in controls (68,2mg, $p = 0,001$), but no difference in brain weight was established in the second experiment.

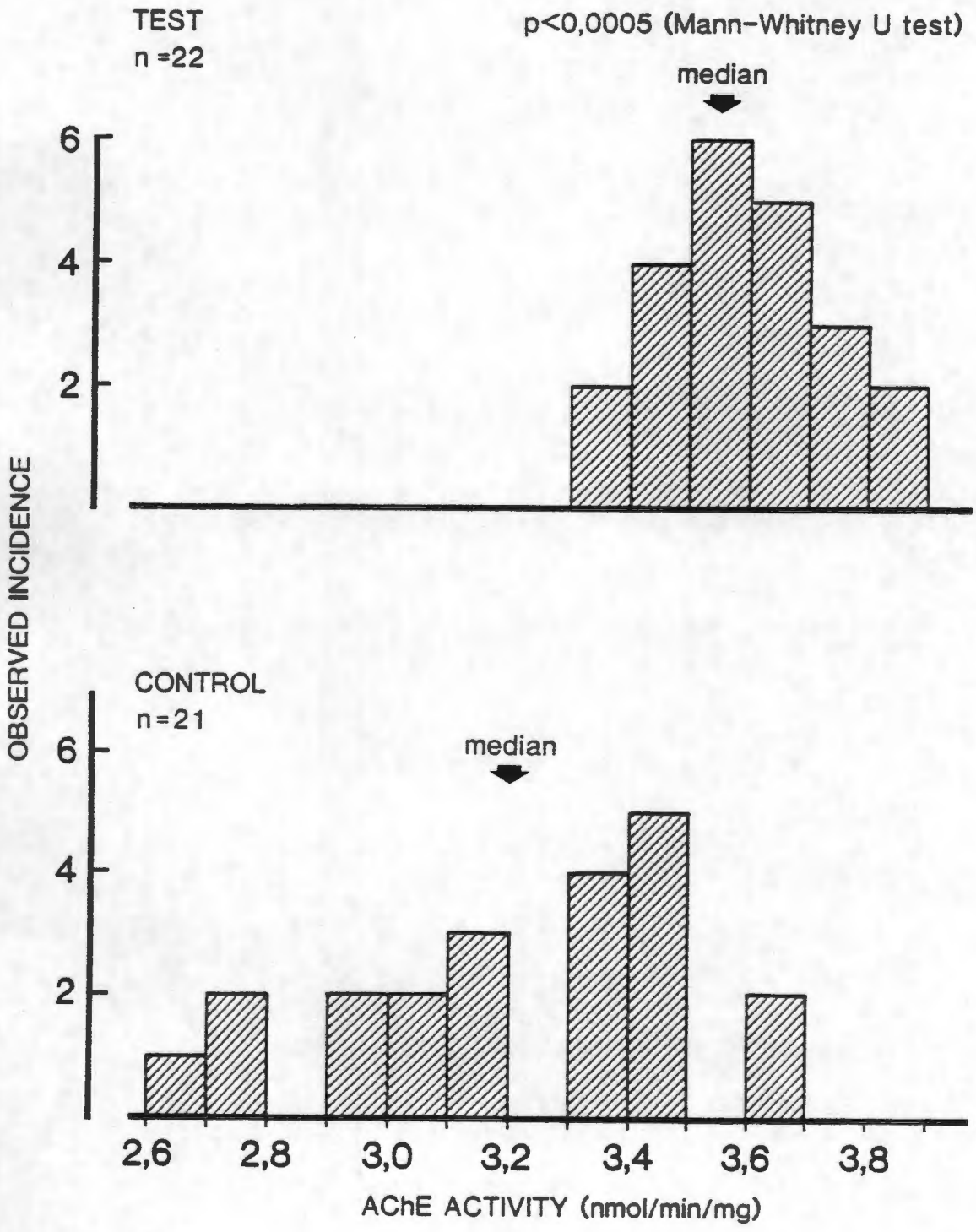
In the first experiment the median brain AChE activity in the treated fetuses of 3,20nmol/min/mg was significantly higher than in controls (2,99nmol/min/mg, $p = 0,005$). In the second experiment median brain AChE activities of test and control fetuses were 3,55 and 3,19nmol/min/mg respectively ($p<0,0005$). The difference in AChE activity in the test and control groups in the second experiment is demonstrated graphically in Fig. 6.8. The well defined population of treated fetuses with higher brain AChE activity is apparent.

Discussion

The fact that there was no established increase in incidence of resorp-

Fig. 6.8. AChE ACTIVITY IN FETAL MOUSE BRAIN

(10000 IU VITAMIN A ADMINISTERED 10 DAYS p.c.)



tions or intra-uterine deaths in the treated group compared with controls demonstrated that a dose of 10 000 IU vitamin A administered on day 10 was not embryolethal. (However, subsequent experiments showed that smaller doses administered 1 to 3 days earlier could be embryolethal, resulting in a 100% resorption rate).

The high incidence of gross malformations (42,3% and 54,5%) respectively compared with none in controls, confirmed the teratogenicity of this dose and time of administration of vitamin A, as was noted in preliminary experiments (section 6.3.5.)

There was no proven difference in fetal weights between the test and control groups and this suggested that 10 000 IU vitamin A on day 10 p.c. did not have an effect on fetal growth. It also indicated that embryotoxicity associated with maternal nutritional deprivation of the fetus, either at a placental or other level, was unlikely. In the face of no significant difference in fetal weights the significantly higher brain weights in the test group in the first experiment is difficult to explain (in the second experiment there was no significant difference in brain weights).

The significant increase in brain AChE activity in the treated group in both experiments may have been the result of a teratogenic effect of vitamin A on the CNS. It was unlikely that this increase in AChE was related to an effect on fetal growth since no difference in fetal weights of treated fetuses compared with controls was established. However, it is possible that the increase in AChE was related in part to an increase in brain weight because brain weight was significantly increased in the first experiment, and the median brain weight (86,65mg) was greater than controls (84,6mg) in the second experiment although this was not significant.

Acetylcholinesterase is a protein and the possibility that its increase was a reflection of a general increase in brain protein content, which may have accounted for the increased brain weight, was considered and pursued in section 6.5.

Another potential mechanism for the increase in AChE activity was relative sparing of cholinergic neurons from the CNS damage of the teratogen, or more rapid repair of cholinergic neurons, compared with other neuronal cell types such as neuroblasts. Investigations with another enzyme involved in the cholinergic system, choline acetyltransferase were performed in an attempt to help elucidate this possibility (see section 6.6).

To determine whether the increase in AChE activity was associated with a particular isoenzyme, the isoenzymes of AChE were analysed (see section 6.7).

The increased AChE cannot be explained on the basis of increased blood in the brain (compare 12 day fetuses treated with cyclophosphamide - see Chapter 7). Milligram for milligram the blood of 18-day fetuses has a substantially lower AChE activity than brain and any increase in blood, for example with haemorrhagic necrosis would be expected to <u>decrease</u> AChE.

Having demonstrated that a significant increase in brain AChE was produced by administration of 10 000 IU vitamin A on day 10 p.c. the effect of administration <u>later</u> in gestation, during the period of fetogenesis when the CNS is less likely to be sensitive to teratogenic insults, and the effect of 10 000 IU or <u>smaller</u> doses of vitamin A administered on one or more days <u>earlier</u> in gestation, when the CNS is likely to be more vulnerable, was determined.

6.4.6. Vitamin A, 10 000 IU per mouse, administered on day 13 p.c. (sacrifice day 18)

Vitamin A 10 000 IU, which had now been shown to be teratogenic and associated with a change in brain AChE when administered on day 10 p.c., was administered later in gestation on day 13 p.c.

See Tables 6.29T and 6.29C for original data (pp. 369-370).

Summary of results in Table 6.30

There were no resorptions, two intra-uterine deaths in the test group and one in the control group, and no gross abnormalities in either group. There was no significant difference in fetal weight, brain weight or brain AChE activity.

Discussion

The absence of any significant differences between test and controls suggested an absence of any teratogenic effect. This is in keeping with the concept that the fetus is less susceptible to embryotoxic effects when exposed later in gestation.

6.4.7. Vitamin A, 10 000 IU per mouse, administered on day 9 p.c. (sacrifice day 19)

See Tables 6.31T and 6.31C for original data (pp. 371-373).

Summary of results in Table 6.32.

This experiment was performed to compare the effects of dosing a day earlier during the period of brain development.

There were four resorptions in the test group and none in the control group ($p>0,1$), two intra-uterine deaths in the test group and none in the control group. All the fetuses in the treated group were abnormal.

T A B L E 6.30.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 13 p.c.

	TEST	CONTROL	p
Total Conceptions	21	22	-
Resorptions (%)	0	0	-
Intra-uterine Deaths (%)	9,5	4,5	NS
Abnormalities (%)	0	0	-
Median Fetal Weight (g)	0,985 (0,94 - 1,03)	0,97 (0,92 - 1,03)	NS
Median Head Weight (mg)	74,2 (71,0 - 76,8)	71,8 (68,5 - 74,2)	NS
Median AChE Activity nmol/min/mg	2,48 (2,37 - 2,60)	2,44 (2,37 - 2,53)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

T A B L E 6.32.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 9 p.c.

	TEST	CONTROL	p
Total Conceptions	27	19	-
Resorptions (%)	14,8	0	NS
Intra-uterine Deaths (%)	7,4	0	NS
Abnormalities (%)	100	0	<0,001
Median Fetal Weight (g)	1,16 (1,10 - 1,22)	1,25 (1,2 - 1,32)	0,055
Median Brain Weight (mg)	78,40 (74,75 - 82,1)	82,73 (80,55 - 84,5)	0,067
Median AChE Activity nmol/min/mg	3,375 (3,30 - 3,47)	3,375 (3,30 - 3,45)	NS

Figures in brackets are the 95% confidence range
For statistical tests used see Section 6.2.3.

The main abnormalities were exophthalmos and tail defects. Two fetuses had spina bifida. The median fetal weight of test animals (1 160mg) was lower than that of controls (1 250mg $p = 0,055$). Median brain weight was also lower for tests (78,4mg) compared with controls (82,73mg, $p = 0,067$). There was no significant difference in brain AChE activity in the treated group.

Discussion

In the face of an increased AChE activity with 10 000 IU vitamin A administered on day 10, the absence of any significant difference in AChE activity in treated animals compared with controls in this experiment was at first sight surprising, especially in view of the higher incidence of gross morphological abnormalities, which suggested a more pronounced teratogenic effect of the vitamin when administered on day 9. However, the lower fetal and brain weights in the treated group suggested some growth inhibitory effect which is consistent with the findings of others (Kochar, 1968; Morriss and Steele, 1974; 1978). This would be expected to be associated with a lower AChE activity (see cyclophosphamide experiments, Chapter 7) but could have been offset by another effect of vitamin A, which in the previous experiment was associated with an increase in enzyme activity, the net result reflecting no significant change.

Alternatively, just as dysmorphogenic manifestations on day 10 are different to those on day 9, biochemical changes might also differ. If biochemical repair in the brain was complete by the time of sacrifice (despite the persistence of other gross morphological abnormalities) no change in AChE activity would have been anticipated. To attempt to determine whether the absence of any significant change in AChE in the previous experiment was due to repair (and return to normal of the enzyme by the time of sacrifice), fetuses were examined earlier on the 12th day post conception in the following two experiments.

The possibility also exists that changes in enzyme activity may vary in different parts of the brain, such that an increase in one area may be offset by a decrease in another, resulting in no net change in total brain activity. Specific brain areas were therefore examined (see sections 6.4.9. and 6.4.10).

6.4.8. Vitamin A, 10 000 IU per mouse, administered on day 9 p.c. (sacrifice day 12)

See Tables 6.33T and 6.33C (pp. 374 and 375) for original data of the first experiment.

See Tables 6.35T and 6.35C (pp. 376 - 378) for original data of second experiment.

Summary of results in Tables 6.34. and 6.36.

There was no difference in the incidence of resorptions in either experiment. No intra-uterine deaths or fetal abnormalities were noted in the first experiment. In the second experiment there were three IUD's in the one pregnancy with haemorrhagic, necrotic fetal tissue and blood stained amniotic fluid, and in three of the viable fetuses abnormal shaped heads were noted and one fetus had a hypoplastic face. These features were all consistent with a teratogenic effect of vitamin A. There was no significant difference in fetal weight, brain weight or head AChE activity between tests and controls in either experiment.

Discussion

These results tended to exclude the possibility that the absence of any significant difference in head AChE activity in treated fetuses compared with controls in the previous experiment was due to repair, since repair is less likely to be complete by day 12 compared with day 18. However, repair appears to be a rapid process and the possibility exists that it

T A B L E 6.34.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 9 p.c. (SACRIFICE DAY 12 p.c.) (FIRST EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	24	24	-
Resorptions (%)	0	4,2	NS
Intra-uterine Deaths (%)	0	0	-
Abnormalities (%)	0	0	-
Median Fetal Weight (g)	0,075 (0,07 - 0,078)	0,073 (0,067 - 0,078)	NS
Median Brain Weight (mg)	30,5 (29,2 - 32,3)	28,05 (25,3 - 31,0)	NS
Median AChE Activity nmol/min/mg	0,669 (0,64 - 0,72)	0,718 (0,65 - 0,80)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

TABLE 6.36.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 9 p.c. (SACRIFICE DAY 12 p.c.) (SECOND EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	23	21	-
Resorptions (%)	0	0	-
Intra-uterine Deaths (%)	13,0	4,8	NS
Abnormalities (%)	20	0	<0,05
Median Fetal Weight (g)	0,07 (0,066 - 0,072)	0,073 (0,069 - 0,078)	NS
Median Head Weight (mg)	23,38 (21,05 - 26,05)	25,65 (23,9 - 27,65)	NS
Median AChE Activity nmol/min/mg	0,761 (0,68 - 0,82)	0,845 (0,76 - 0,93)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

could have been accomplished within 72 hours (Langman et al., 1980). Because of the technical difficulty in assessing brain or head AChE in younger fetuses this avenue was not pursued further.

6.4.9. Vitamin A, 10 000 IU per mouse, administered on day 9 p.c. (sacrifice day 18 p.c.) Determination of cerebellar AChE activity

Two experiments were performed.

See Tables 6.37T, 6.37C, 6.39T and 6.39C for original data (pp. 379-384).

Summary of results in Tables 6.38. and 6.40.

The cerebellum was dissected free in toto from adjoining brain with a small spatula and weighed, solubilised and homogenised as for whole brain (section 2.8).

No difference in the incidence of resorptions or intra-uterine deaths in the treated group was proven in either experiment compared with controls. There was a 85,7% and 100% incidence of malformations respectively compared with 0% in controls. Median fetal weights of test fetuses were significantly lower (1,026g and 0,930g) than controls (1,086g and 1,036g, $p<0,02$ and 0,003 respectively), although there was no significant difference in brain weight, cerebellar weight or cerebellar AChE activity of vitamin A-treated fetuses compared with controls.

Discussion

See discussion in section 6.4.10.

6.4.10. Vitamin A, 10 000 IU per mouse, administered on day 9 p.c. (sacrifice day 18 p.c.) Determination of AChE activity in cerebral cortex

See Tables 6.41T and 6.41C for original data (pp. 385-387).

T A B L E 6.38.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 9 p.c. (SACRIFICE DAY 18) FIRST EXPERIMENT

	TEST	CONTROL	P
Total Conceptions	25	23	-
% Resorptions	4,0	4,3	N.S.
% Intra-uterine Deaths	12,0	0	< 0,001
% Abnormalities	85,7	0	< 0,001
Median Fetal Weight (g)	1,026 (0,92-1,14)	1,086 (1,03-1,16)	< 0,02
Median Brain Weight (mg)	69,4 (57,1-79,2)	70,6 (63,2-76,1)	N.S.
Median Cerebellar Weight (mg)	21,9 (17,6-26,4)	22,5 (18,7-26,3)	N.S.
Median Cerebellar AChE Activity nmol/min/mg	4,24 (3,7-5,5)	4,28 (3,8-4,6)	N.S.

TABLE 6.40.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 9 p.c. (SACRIFICE DAY 18) SECOND EXPERIMENT

	TEST	CONTROL	P
Total Conceptions	22	20	
% Resorptions	0	0	N.S.
% Intra-uterine Deaths	4,5	0	N.S.
% Abnormalities	100	0	< 0,001
Median Fetal Weight (g)	0,930 (0,79-1,06)	1,036 (0,9 - 1,11)	0,003
Median Brain Weight (g)	69,1 (60,4-75,4)	74,1 (67,5-78,8)	N.S.
Median Cerebellar Weight (mg)	25,0 (21,5-28,4)	27,0 (25,0-30,0)	N.S.
Median Cerebellar AChE Activity nmol/min/mg	3,98 (3,5-4,53)	4,01 (3,6-4,63)	N.S.

Summary of results in Table 6.42.

Using blunt dissection the cerebral cortices were separated from the underlying corpus callosum and adjacent brain, and weighed, solubilised and homogenised as for whole brain (section 2.8). There was no significant difference in the incidence of resorptions or intra-uterine deaths compared with controls. Median fetal weights of test fetuses were significantly lower than controls (0,975g and 1,041g respectively, p 0,006), although there was no significant difference in brain weight or cerebral cortical weight or AChE activity compared with controls.

Discussion

The specific brain areas used were the cerebellum and cerebral cortex owing to the small size of fetal mouse brains and because these are ontogenetically newer CNS areas than, for example, the mid brain or brain stem, which are more likely to be sensitive to teratogenic insults.

The absence of a change in whole brain AChE activity in the presence of differences in specific brain areas has been noted by other workers. Eckhert, Barnes and Levitzky (1976) found changes in rat forebrain and brain stem AChE activity in opposite directions which cancelled out one another, resulting from undernutrition during development. Furthermore, the effect of another teratogenic agent, X-radiation on rat brain AChE activity was found to depend on the brain region, the cerebellum showing more marked alteration than the cortex (Valcana and Timiras, 1974). In the present study, however, a teratogenic dose of vitamin A was not associated with a change in AChE activity in either the cortex or the cerebellum and the absence of a change in the whole brain in section 6.4.7. cannot be ascribed to a differential effect in these brain regions.

T A B L E 6.42.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 9 p.c. (CORTEX EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	23	21	-
Resorptions (%)	0	0	NS
Intra-uterine Deaths (%)	4,3	4,8	NS
Abnormalities (%)	100	0	<0,001
Median Fetal Weight (g)	0,975 (0,929 - 1,014)	1,041 (1,009 - 1,068)	0,006
Median Cortex Weight (mg)	29,1 (27,4 - 30,6)	28,1 (25,9 - 31,0)	NS
Median AChE Activity nmol/min/mg	0,557 (0,51 - 0,61)	0,539 (0,48 - 0,61)	NS

Figures in brackets are the 95% confidence range

The AChE activity of cerebellum of approximately 4nmol/min/mg was higher than that in the cortex or the whole brain. The relative distribution of AChE activity varies with species, strain and age. In the adult rat the highest activity is in the brain stem followed in decreasing order by the hypothalamus, cerebellum and cerebral cortex (Maletta and Timiras, 1966; Vijayan and Brownson, 1974), although the cerebellum is usually reported to have the lowest activity (Maletta, Vernadakis and Timiras, 1967). However, in 18-day rat fetuses the highest AChE activity was noted in the cerebellum with the lowest in cerebral cortex (Maletta, Vernadakis and Timiras, 1967), which is in agreement with the findings of this study.

The low activity in the cerebral cortex suggests a small cholinergic component of this region in the mouse fetus. This exposes a potential limitation of AChE as a brain marker of teratogenic injury in the mouse fetus since the ontogenetically new cerebral cortex would be expected to be particularly susceptible to teratogenic insult.

Further experiments with different vitamin A dosing regimens on days 7, 8 and/or 9, (when the CNS is particularly susceptible to teratogenic insults)were next performed with a view to comparing the effects of longer exposure to the teratogen with those obtained in the previous experiments.

6.4.11. Vitamin A, 5 000 IU per mouse, administered on each of days 8 and 9 p.c. (sacrifice day 19)

See Table 6.43. for original data (p. 388).

Five dams were used in test group. There were 35 resorptions and no live fetuses. Controls were not sacrificed in this experiment.

Discussion

Five thousand IU administered on each of days 8 and 9 p.c. was therefore embryolethal and unsuitable for study of brain AChE.

6.4.12. Vitamin A, 7 500 IU per mouse, administered on day 8 p.c. (sacrifice day 19)

See Table 6.44. for original data (p. 389).

Five dams were used in the test group. There were 36 resorptions and one intra-uterine death with blood stained hydramnios (the fetus was exencephalic). There were no live fetuses. Controls were not sacrificed in this experiment.

Discussion

Seven thousand five hundred IU vitamin A administered on day 8 p.c. was therefore also embryolethal and unsuitable for study of brain AChE.

6.4.13. Vitamin A, 2 500 IU per mouse, administered on each of days 7, 8 and 9 p.c. (sacrifice day 18)

See Table 6.45T and 6.45C for original data (pp. 390 and 391).

Four dams were used in the test group. There were 22 resorptions and only five live fetuses, one of which had exencephaly. There were no resorptions in the control group, one intra-uterine death and 20 live fetuses.

Discussion

This regimen was also largely embryolethal and was unsuitable for study of brain AChE because the number of fetuses would have been too small for meaningful statistical analysis.

6.4.14. Vitamin A, 2 500 IU per mouse, administered on each of days 7 and 8 p.c. (sacrifice day 19)

See Table 6.46 for original data (p. 392).

Four dams were used in the test group. There were 21 resorptions and only five live fetuses, one of which had anophthalmia.

Discussion

This regimen was also largely embryolethal and was also unsuitable for study of brain AChE because of the small number of fetuses. Controls were not sacrificed in this experiment.

6.4.15. Vitamin A, 3 000 IU per mouse, administered on each of days 8 and 9 p.c.

See Tables 6.47T and 6.47C for original data (pp. 393 and 394).

Summary of results given in Table 6.48.

Five thousand IU administered on days 8 and 9 p.c. was found to be embryolethal (section 6.4.11). A smaller dose of 3 000 IU was therefore used in this experiment to enable comparison with results obtained with dosing on day 9 alone and on day 10.

There were five resorptions in the treated group and none in the control group ($p<0,05$), and one intra-uterine death in each group. Three gross abnormalities were present in the test group (one fetus with exencephaly and two with exophthalmos) and one in the control group with exomphalos. The median test fetal weight of 1 063mg was significantly lower than that of the control group (1 149mg, $p = 0,006$). Similarly, the median brain weight of the test group (74,9mg) was significantly lower than controls (78,4mg $p = 0,01$). However, the median AChE activity for the test group (2,73nmol/min/mg) was higher than that of controls (2,68) although this was not significant.

T A B L E 6.48.

SUMMARY OF RESULTS OF 3 000 IU VITAMIN A ADMINISTERED ON DAYS 8 AND 9 p.c.

	TEST	CONTROL	p
Total Conceptions	21	20	-
Resorptions (%)	19,2	0	<0,05
Intra-uterine Deaths (%)	4,8	5,0	NS
Abnormalities (%)	14,3	5	NS
Median Fetal Weight (g)	1,063 (0,96 - 1,09)	1,149 (1,12 - 1,18)	0,006
Median Brain Weight (mg)	74,9 (68,4 - 77,3)	78,4 (76,4 - 80,9)	0,01
Median AChE Activity nmol/min/mg	2,73 (2,65 - 2,81)	2,68 (2,63 - 2,72)	0,2

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

Discussion

The characteristic gross morphological abnormalities noted in three of the fetuses confirmed that this dosing regimen was teratogenic. The lower fetal weights in the treated group also supported this and were in keeping with a growth inhibitory effect, as was found with a dose of 10 000 IU on day 9 p.c. The absence of any significant difference in AChE activity between test and controls may be explicable on the same basis as with dosing with 10 000 IU on day 9.

6.5. THE EFFECT OF VITAMIN A ON FETAL BRAIN TOTAL PROTEIN CONTENT

6.5.1. Introduction

This experiment was performed to help elucidate the mechanism for increased fetal brain AChE activity with a dose of 10 000 IU vitamin A on day 10 p.c. Acetylcholinesterase is a protein and its increase may have merely been a reflection of a general increase in protein content (the significantly increased median brain weight in the test group in one experiment may have suggested this). Brain total protein content was therefore determined and compared with that of controls.

6.5.2. Method

The method used was a modification of the Lowry method (Lowry et al., 1951) by G.L. Peterson (1977, 1983). A standard curve was constructed using a known concentration range (5 - 100µg) of a standard protein (bovine serum albumin - BSA).

An assay using spiked samples was first performed. Ten standard samples, containing 5 - 100µg of BSA protein (10 - 200µl of a 0,5mg/ml solution) were made up to 1ml with distilled water. 1,0ml of "reagent A" (one part copper-tartrate-carbonate: 0,1% $CuSO_4.5H_2O$, 0,2% sodium tartrate, 10%

Na_2CO_3; 2 parts 5% sodium dodecyl sulphate (SDS); 1 part 0,8M NaOH) was added to each standard, the solution mixed and allowed to stand at room temperature for 10min. 0,5ml of "reagentB" (1 part 2N Folin-ciocalteu phenol reagent + 5 parts distilled water) was then added to each standard, mixed immediately and allowed to stand at room temperature for 30min. Absorbance was read at 750nm against a standard blank containing 1ml distilled water with no BSA.

Assay of experimental samples was then performed. Fetal mouse brain was homogenised in 0,05M Tris-HCl (pH 8,4), containing 0,5% Triton X-100 to give a 30mg/ml solution. Samples containing 15µl of homogenate were made up to 1ml with distilled water. 0,1ml of 0,15% sodium deoxycholate was added to each sample, the solution mixed and allowed to stand for 10 min at room temperature. 0,1ml of 72% trichloroacetic acid was then added and each sample centrifuged for 5 - 30min at 1 000 - 3 000g. After centrifugation, the supernatant was decanted and any remaining supernatant removed using a pasteur pipette attached to a vacuum pump. One ml of reagent A was added directly to the precipated protein and the assay performed in the same way as described under standard assay. The sample blank contained 15µl of sample buffer made up to 1,0ml with distilled water.

6.5.3. Lowry Total Protein Determination : Results of 10 000 IU vitamin A per mouse administered on day 10 p.c. (sacrifice day 18 p.c.)

Fetal brain total content was measured after maternal administration of 10 000 IU vitamin A on day 10 p.c. and compared with controls to determine whether the increase in brain AChE noted in section 6.4.5. was a reflection of a general increase in brain protein.

See Tables 6.49C and 6.49T for original data (pp. 176 and 177).

T A B L E 6.49C.

LOWRY TOTAL PROTEIN DETERMINATION : CONTROLS

Sample No.	Fetal weight (mg)	Brain weight (mg)	mg protein/ mg brain
C1	1141,2	78,1	0,089
C2	1071,1	75,1	0,084
C3	1119,3	77,9	0,089
C4	1015,4	69,8	0,080
C5	1208,2	75,7	0,076
C6	992,3	74,6	0,087
C7	968,6	71,7	0,084
C8	1065,0	74,0	0,080
C9	1022,7	70,7	0,087
C10	1077,5	74,6	0,091
C11	1100,2	76,2	0,096
C12	1041,8	71,2	0,084
C13	1062,3	76,6	0,078
C14	1123,1	75,8	0,084
C15	1086,1	71,8	0,091
C16	1133,9	72,3	0,096
C17	1113,4	75,9	0,084
C18	1099,6	72,0	0,091
C19	1112,4	76,4	0,087
C20	1053,0	68,7	0,098
Median	1082,0	73,95	0,087
95% Confidence limits	1053 - 1107	72,65 - 75,5	0,084 - 0,090

T A B L E 6.49T.

LOWRY TOTAL PROTEIN DETERMINATION : 10 000 IU VITAMIN A PER MOUSE ON DAY 10 p.c. (SACRIFICE DAY 18 p.c.)

Sample No.	Fetal weight (mg)	Brain weight (mg)	mg protein/ mg brain
T1	1084,9	75,9	0,067
T2	1060	75,7	0,076
T4	1035,3	76,2	0,064
T6	1043,9	73,9	0,069
T7	968,3	72,4	0,084
T8	1001,2	74,6	0,087
T9	1065,5	79,0	0,078
T10	1101,5	80,7	0,1089
T11	1108,5	80,2	0,082
T12	1041,0	76,1	0,100
T13	1028,0	75,1	0,082
T14	966,4	74,7	0,087
T15	1205,9	77,3	0,084
T16	1059,7	70,8	0,098
T17	1200,6	85,0	0,087
T18	1053,0	75,5	0,084
T19	1131,9	79,4	0,087
Median	1063,0	76,20	0,084
95% Confidence limits	1034 - 1102	74,9 - 78,15	0,077-0,090
p. *	NS	NS	NS

* Comparison with data in Table 6.49C.

There was no significant difference in fetal weights, brain weights or brain total protein content between tests and controls. For treated fetuses the median brain protein content was 0,084mg protein/mg brain and for controls 0,087mg.

Discussion and conclusion

Maternal administration of 10 000 IU vitamin A on day 10 p.c. was not associated with any change in brain total protein content in 18 day fetuses compared with controls. (As no trend for any meaningful difference was apparent the experiment was not repeated). The increase in brain AChE activity observed in section 6.4.5. therefore does not appear to be a reflection of a general increase in brain total protein content. This is also in keeping with the findings of other workers. Benett et al., (1958a; 1961) were unable to find a positive correlation between AChE and brain protein. Similarly, prenatal X-irradiation did not alter brain protein content in rats although there was a significant change in AChE activity compared with controls (Maletta, Vernadakis and Timiras, 1967).

6.6. THE EFFECTS OF VITAMIN A ON FETAL MOUSE BRAIN CHOLINE ACETYLTRANSFERASE ACTIVITY

6.6.1. Introduction

In contrast to AChE which is responsible for the brakdown of acetylcholine, choline acetyltransferase (ChAT, E.C.2.3.1.6.) catalyses the synthesis of acetylcholine from acetyl-CoA and choline. Choline acetyltransferase is found almost exclusively in neural tissue in cholinergic neurons. The activity of the enzyme correlates well with the concentration and synthesis of acetylcholine (Kobayoshi et al., 1975) and has been used as an enzymic marker for cholinergic cells (Glover and Green, 1972).

Because ChAT is also closely involved with acetylcholine metabolism the aim of this study was:

i) To compare the effects of vitamin A on ChAT activity with those on AChE activity;

ii) to compare ChAT as a potential biochemical marker of teratogenic injury with AChE;

iii) to help elucidate the teratogenic mechanism of action of vitamin A. For example, if the increase in AChE activity with dosing on day 10 of gestation was due to stimulation of cholinergic synthesis or exuberant repair then a concomitant increase in ChAT would be anticipated. However, if the elevation in AChE was due to a relative sparing of cholinergic neurons, ChAT might not be expected to be increased.

Other workers have compared ChAT and AChE activity in rat brain. Valcana and Timiras (1974) studied the effects of X-irradiation; Eckhert et al., (1976) studied the effects of undernutrition, whilst Johnson et al (1984) studied the teratogenic effects of procarbazine. Also, there is a slow continuous rise in ChAT activity in brain tissue during development (Bus and Gibson, 1974). Any perturbation of normal development could therefore be anticipated to alter ChAT activity.

A radiochemical assay of ChAT was used based on that of Fonnum (1966; 1975) which is reproducible, rapid and specific. The method involves the formation of labelled acetylcholine from labelled acetyl-CoA. ^{14}C acetyl-CoA reacts with choline iodide (ChI) in the presence of ChAT to give ^{14}C acetylcholine.

$$^{14}C\ \text{AcCoA} + \text{ChI} \xrightleftharpoons{\text{ChAT}} {}^{14}C\ \text{ACh} + \text{CoA}$$

The labelled ACh is separated from the radioactive substrate by liquid cation exchange using sodium tetraphenylboron (Kalignost) in acetonitrile with a scintillation mixture acting as extraction solvent, and then determined by liquid scintillation counting.

6.6.2. Materials and Methods

Brains were homogenised with a Thomas teflon pestle homogeniser in 10mM EDTA, (pH 7,4), containing 0,5% Triton X-100 to ensure total release of enzyme activity (Fonnum, 1966).

The incubation mixture (prepared fresh) contained (final conc.): 0,2mM 1-^{14}C acetyl CoA (Weil organisation, Amersham; specific activity 55mCi/mmol); 300mM NaCl; 8mM choline iodide; 20mM EDTA; 0,1mM physostigmine sulphate; 50mM sodium phosphate buffer (pH 7,4). The concentrations of acetyl-CoA and choline iodide used saturate the enzyme. The pH was adjusted to simulate intracellular conditions (pH 7,4) although ChAT is most active at pH 8,0 (Glover and Green, 1972). Since the enzyme is inhibited by trace elements of copper, EDTA is added. Physostigmine is added to inhibit AChE, cholinesterases and simple esterases which can break down acetylcholine.

Two microlitres brain homogenate and 5µl incubation mixture were placed in a microtube. The solution was mixed with a rotamixer for a few seconds and incubated for 15min at 37°C in a water bath. The reaction was then stopped by immersing the microtube in crushed ice. The contents of the microtube were flushed into a glass scintillation vial with 3ml of 10mM sodium phosphate buffer (pH 7,4). (If the tube was left in the vial for the remainder of the experiment, as described by Fonnum (1975), higher background counts were obtained). To this was added 2ml acetonitrile containing 10mg sodium tetraphenylborate (Merck) and 10ml of toluene scintillation cocktail (0,05% diphenyloxazole and 0,02% 1,4-bis-(4-methyl-5-phenyloxazole-2-yl) benzene). The vial was shaken gently for 15 to 20 sec, enabling labelled ACh to be extracted into the toluene or organic phase, whereas the acetyl-CoA is left in the aqueous phase. The mixture was allowed to stand for 30min which resulted in separation into an upper

organic and lower aqueous phase. Nine millilitres of the upper toluene phase (with the labelled ACh) were decanted into a plastic scintillation vial and counted for 10min in a Packard TriCarb 4640 liquid scintillation counter. Each sample was assayed in duplicate. (Note : i) Fonnum (1975) did not separate the phases for counting. In this study, however, it was found that this resulted in no difference in counts for reactions with or without ChAT, which suggested that both phases were being counted. ii) As was noted in the technique of Glover and Green (1972), some labelled acetyl-CoA is also extracted into the organic layer and gives rise to substantial blank counts. Spontaneous formation of ACh can presumably also occur).

6.6.3. Relationship between choline acetyltransferase activity and fetal mouse brain concentration

This experiment was performed to determine whether there was a satisfactory relationship between brain concentration (and hence ChAT content) and measured ChAT activity.

Materials and Methods

Brains were homogenised with a Thomas teflon pestle homogeniser in 10mM EDTA (pH 7,4), containing 0,5% Triton X-100 to give brain concentrations of 15,30 and 60mg/ml. A fourth sample (the blank) contained no brain. The radio-chemical assay, described in the previous section, was used to determine ChAT activity as disintegrations per minute, d.p.m. Five or six samples were tested for each brain concentration.

Results are given in Table 6.50. and the relationship between ChAT activity (d.p.m.) and brain homogenate concentration shown graphically in Fig. 6.9.

T A B L E 6.50.

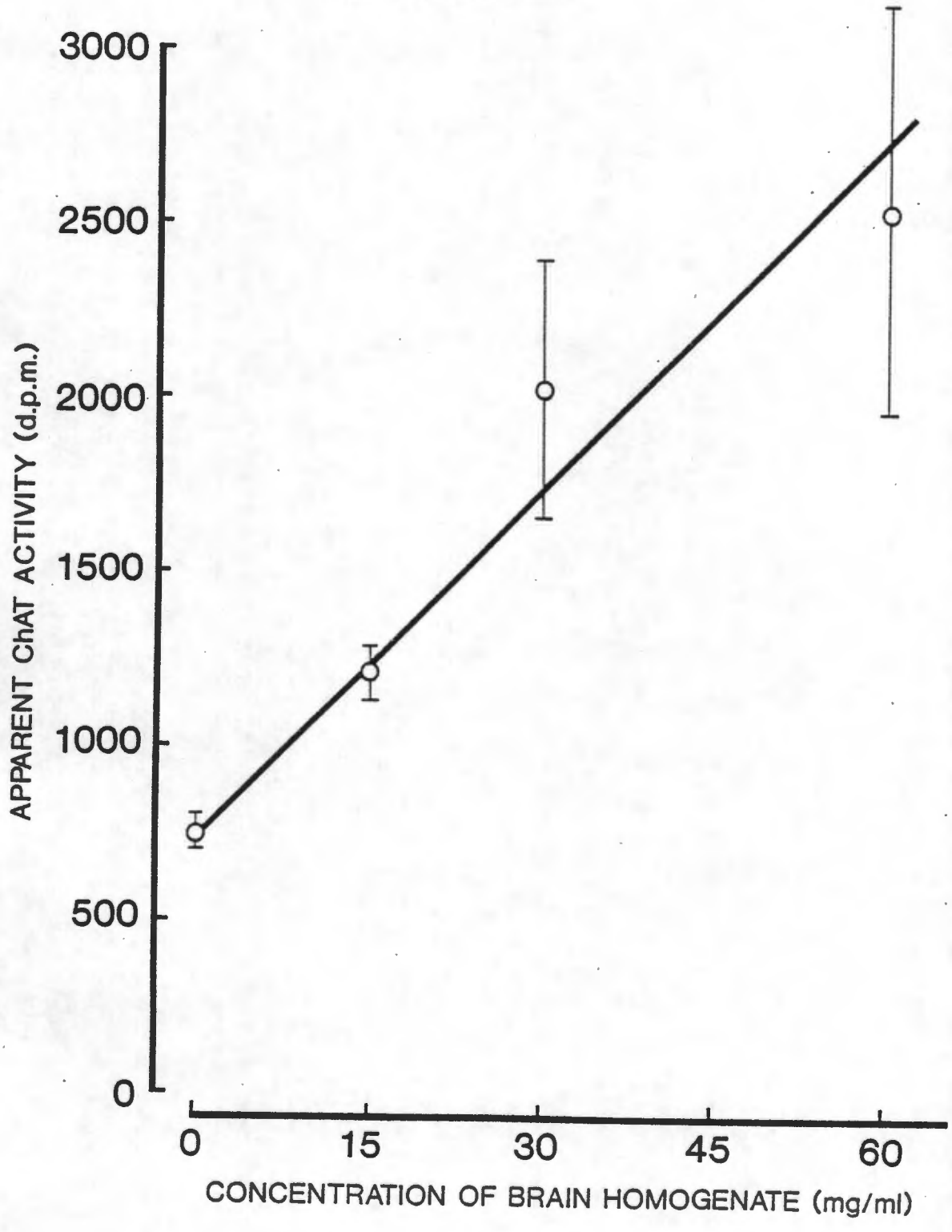
OBSERVED CHOLINE ACETYLTRANSFERASE ACTIVITY IN d.p.m. FOR DIFFERENT CONCENTRATIONS OF BRAIN HOMOGENATE

Brain Concentration (mg/ml)	d.p.m.	mean	Coefficient of variation %	S.D.
0	797 802 726 740 702 706	745	6	44
15	1280 1152 1292 1199 1121	1209	6	76
30	1709 1907 2663 1870 1967	2023	18	370
60	3631 2542 2312 2244 2400 1985	2526	23	574

Spearman r = 0,878 $\equiv$ t = 8,18; p $<<$ 0,001

Fig. 6.9.

RELATIONSHIP BETWEEN ChAT ACTIVITY (d.p.m.) AND BRAIN HOMOGENATE CONCENTRATION (mg/ml)



Error bars are 95% confidence limits.

Conclusions

a) A linear relationship is apparent between brain concentration (and hence ChAT content) and ChAT activity ($p < 0,001$).

b) The results indicate substantial ChAT activity.

c) High blank readings are apparent but are acceptable in view of the substantially higher counts when ChAT is present.

6.6.4. Vitamin A, 10 000 IU per mouse, administered on day 10 p.c.

This experiment was performed to compare the effects on ChAT with those on AChE activity.

See Tables 6.51T and 6.51C for original data (pp. 395 and 396).

Summary of results in Table 6.52.

There was no significant difference in the incidence of resorptions or intra-uterine deaths compared with controls. All treated fetuses had gross abnormalities in that the tails were short or kinked, compared with only one abnormal control fetus with exomphalos ($p<0,001$). There was no significant difference in fetal weight, brain weight or whole brain ChAT activity of treated fetuses compared with controls.

Comment

The above results are consistent with those of previous experiments (section 6.4.5.), except that, in contrast to AChE activity which was greater in treated fetuses, there was no significant difference in ChAT activity compared with controls. Discussion regarding the absence of any change in ChAT activity is presented later - see section 6.6.6.

6.6.5. Vitamin A, 15 000 IU per mouse, administered on day 10 p.c.

With no significant difference in brain ChAT activity with 10 000 IU vitamin A on day 10 this experiment was performed to determine the effects

T A B L E 6.52.

SUMMARY OF RESULTS OF 10 000 IU VITAMIN A ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 19 p.c.)

	TEST	CONTROL	p
Total Conceptions	20	23	-
Resorptions (%)	0	8,7	NS
Intra-uterine Deaths (%)	5,0	4,3	NS
Abnormalities (%)	100	5,0	<0,001
Median Fetal Weight (g)	1,284	1,270	NS
Median Brain Weight (mg)	77,75	76,45	NS
Median ChAT Activity as d.p.m.	775,8	804,8	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

of a larger dose of the teratogen.

See Tables 6.53T and 6.53C for original data (pp. 397-399).

Summary of results in Table 6.54.

There was a marked increase in the incidence of late resorptions (58,9%) with the higher dose compared with none in controls ($p<0,001$). A feature that had not been previously observed was that, although the fetuses had largely resorbed, leaving a $\pm$ 4mm blob of necrotic tissue, the placentae were well developed and of almost comparable size to those of viable fetuses. Of the viable fetuses all had gross abnormalities such as kinked tails and spade-like limbs (a more pronounced effect of the higher dose on limb development was apparent).

The median fetal weight of treated fetuses (0,943g) was lower than that of control fetuses (1 002g, $p = 0,014$) and may have indicated a mild growth inhibitory effect of the higher dose. There was no significant difference in brain weight or brain ChAT activity.

6.6.6. Discussion

The finding of resorbed fetal tissue with well developed placentae was compatible with a direct effect of vitamin A on the fetus, which is in agreement with the observations of others (see section 1.2). The high incidence of late resorptions (58,9%) with 15 000 IU compared with 10 000 IU vitamin A (see section 6.4.5.) demonstrated a dramatic increase in embryolethality with the increment in dose. Similarly, a higher incidence of gross abnormalities (100%) was apparent with the larger dose.

The absence of any change in ChAT activity in the treated group in the above two experiments in the face of the previously demonstrated increase in AChE activity with the same dose and time of administration of vitamin

T A B L E 6.54.

SUMMARY OF RESULTS OF 15 000 IU VITAMIN A

ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 18 p.c.)

	TEST	CONTROL	p
Total Conceptions	39	23	-
Resorptions (%)	58,9	0	<0,001
Intra-uterine Deaths (%)	2,6	8,7	NS
Abnormalities (%)	100	0	<0,001
Median Fetal Weight (g)	0,943	1,002	0,014
Median Brain Weight (mg)	69,25	68,3	NS
Median ChAT Activity as d.p.m.	352	424	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 6.2.3.

A, may suggest that the increase in AChE activity was not secondary to exuberant repair or hyperplasia of cholinergic neurons. Such a process would be expected to be associated with an increase in ChAT, the synthetic enzyme of acetylcholine. The result may rather favour a process such as relative sparing of cholinergic neurons.

The above is speculative for two reasons. Firstly, there was a wide standard deviation in ChAT between different brain homogenates, which would have made detection of more subtle differences in ChAT activity difficult. Secondly, the relationship in activity of cholinergic enzymes is complex such that the activity of one enzyme does not necessarily parallel the other. For example, Eckhert et al., (1976) found that undernutrition imposed during development in the rat could be associated with an increase in AChE activity in a specific brain region, whereas ChAT activity might be unaffected, increased or decreased in the same region. Similarly Valcana and Timiras (1974) found that X-irradiation to the developing rat brain resulted in increased AChE activity in the cortex but no change in ChAT activity.

Because of the absence of any significant trend in ChAT activity in the previous experiments and the problems outlined above, further experiments to determine ChAT activity were not performed. It was evident that this particular enzyme assay was less sensitive than the AChE assay, and it would also appear that ChAT is a poor marker of neuroteratogenicity: Johnson et al., (1984) found no change in brain ChAT activity in the rat after administration of the well established teratogen procarbazine, from day 12 to 15 of gestation, despite causing gross morphological changes including microcephaly.

6.6.7. Conclusions of Choline Acetyltransferase study

a) No significant change in brain ChAT activity has been demonstrated

in C3H mouse fetuses exposed to teratogenic doses of vitamin A on day 10 of gestation.

b) The high standard deviation in ChAT activity between different brain homogenates would have made detection of subtle changes difficult.

c) Using this technique, ChAT would appear to have less potential as a biochemical marker of teratogenic injury than AChE.

d) No definite conclusions can be made from these experiments regarding the teratogenic mechanism of action of vitamin A.

6.7. THE EFFECTS OF VITAMIN A ON FETAL MOUSE BRAIN ACETYLCHOLINESTERASE ISOENZYMES

After maternal administration of 10 000 IU vitamin A on day 10 p.c. mice were sacrificed on day 18 p.c. and the AChE isoenzymes from 20 brain homogenates of treated fetuses were compared with those from 20 controls.

Separation and densitometric analysis of the isoenzymes of AChE were performed as described in Chapter 5.

Results

Five isoenzymes of AChE were present in all test and control samples. No obvious difference was apparent on visual inspection of the gels between tests and controls. Peak heights were measured from the best apparent baseline. The following 3 methods were used in the analysis of the densitometric results obtained.

Method 1: Comparison of peak heights of peaks 1, 2, 3, 4, 5 and the sum of peaks 1 - 5 for test and controls. (The sum of peak heights for peaks 1 - 5 were analysed in order to establish an overall difference between test and controls).

Results are shown in Tables 6.55T and 6.55C (pp. 400 and 401).

Summary of results in Table 6.56.

T A B L E 6.56.

SUMMARY OF DENSITOMETRIC RESULTS FOR VITAMIN A GELS

		Method 1		Method 2		Method 3	
Peaks		Median peak hts mm	P	Median ratio	P	Median ratio	P
1	T	52,75 (n=20)	NS	0,637	NS	0,79	NS
	C	51,88 (n=20)		0,595		0,745	
2	T	98,13	NS	1,184	0,0990	1,47	0,077
	C	96,5		1,095		1,375	
3	T	84,5	NS	-	-	1,243	NS
	C	87,0		-		1,235	
4	T	51,5	NS	0,595	NS	0,75	NS
	C	51,13		0,60		0,722	
5	T	78,6	0,05	0,935	0,032	1,18	0,019
	C	64,88		0,77		0,95	
Sum of 1-5	T	366,8	NS	3,358	0,0124	5,483	0,041
	C	350,0		3,063		4,99	

For derivation of p value see text.

Method 2: Comparison of peak heights of test and controls using one of the peaks as an internal standard in order to attempt to reduce inter- and intra-gel variations in peak height. Peak 3 was chosen as the internal standard because it had the smallest difference in peak height between test and controls. The results for this method were obtained by dividing the peak heights of peaks 1, 2, 4 and 5 by the peak height of peak 3. The ratios for all the peaks were added together as in method 1.
Results are given in Tables 6.57C and 6.57T (pp. 402 and 403).
Summary of results in Table 6.56.
Results are shown graphically in Fig. 6.10.

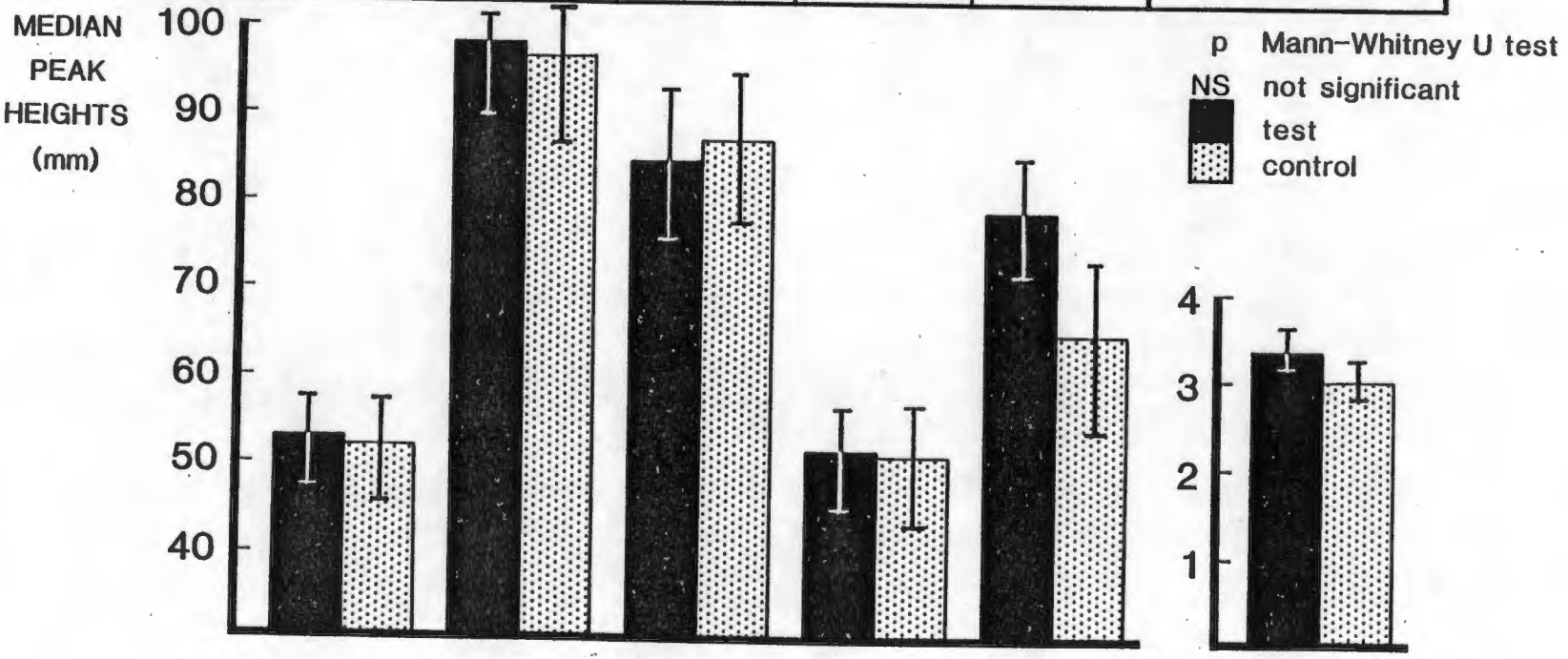
Method 3: Comparison of test and controls in an attempt to minimise inter-gel variation. The peak heights for all the peaks arising from control samples were added together and their mean calculated for each gel. The data for each gel (test and control) was then divided by the appropriate mean.
Results are given in Table 6.58C and 6.58T (pp. 404 and 405).
Summary of results in Table 6.56.
All the results obtained from the 3 methods of analysis were statistically analysed by the Tukey modification of the Willcoxon signed ranks test and the Mann Whitney U test. In addition, some of the peaks were analysed by the Kolmogorov-Smirnov two-sample test which is sensitive both to differences in means and in distribution.

Although the medians for peak heights 1, 2, 5 and the total were greater for tests than controls, analysis of peak heights (Method 1) by Mann Whitney U test only shows a significant difference in peak 5 (median peak height of 78,6mm for treated fetuses compared with 64,9mm in controls, $p<0,05$), but no significant difference in any of the other peaks or in the total (see Tables 6.55C and 6.55T).

Fig. 6.10.

ANALYSIS OF VITAMIN A DENSITOMETER RESULTS

PEAK	1	2	3	4	5	SUM PEAKS 1-5
p	NS	NS	NS	NS	<0.05	0.0124



Inspection of the 95% confidence range suggested the possibility of large differences in peak intensities between gels. Hence Method 2 was employed to try and minimise these variations. Using peak 3 as an internal standard (Method 2) medians for peaks 1, 2 and 5 and the total were again greater for tests, with significant differences for peaks 5 and the total (Median ratio 0,935 and 3,36 respectively compared with 0,77 and 3,06 respectively in controls, p = 0,03 and 0,01: Tukey modifications of the Willcoxon signed ranks test and Mann Whitney U test - see Tables 6.57C and 6.57T.

It is thought, however, that the above procedure might minimise differences in distribution between test and control. Therefore, Method 3 was employed in an attempt to minimise inter-gel variation only. By dividing all the data by the mean control value for each gel, a similar trend as before was observed: the median ratio for band 5 and the total for treated fetuses of 1,18 and 5,48 respectively were higher than for controls (0,95 and 4,99, p = 0,02 and 0,04 respectively), but not for any of the other bands.
Results are shown in Tables 6.58C and 6.58T.

In teratological work it is reasonable to consider that distributions may be bimodal, one mode corresponding to a susceptible subpopulation of tests. The data for peaks 2 and 5 were felt to be particularly likely to give such bimodal distributions and were therefore analysed by the Kolmogorov-Smirnov two-sample test. For peak 2 there was no significant difference between test and control but for peak 5 and the sum of the peaks, a value of p = 0,05, T>C, was obtained. (Data obtained using Method 3).

Discussion and conclusion

Peak 5 and the sum of the peak heights of the isoenzyme bands of AChE

were greater for tests than controls ($p = 0,05$, methods 2 and 3). This was consistent with the significantly higher AChE activity detected for test animals compared with controls using the Ellman technique ($p<0,0005$, section 6.4.5).

The predominant increase in enzyme activity was in band 5 ($p =$ or $<0,05$), although the median peak heights for bands 1 and 2 were also greater for tests but this was not significant.

The absence of any difference in band 4 between tests and controls could simply be accounted for by the faintness of this band.

It was felt unlikely that overcoming minor technical problems such as the high background scatter and inter- and intra-gel variation would substantially improve the sensitivity of the technique, and this avenue of investigation which appeared to be less sensitive than the spectrophotometric assay (see also sections 7.5 and 8.5), was not performed in other experiments with vitamin A where changes in AChE activity were not demonstrable.

6.8. SUMMARY AND DISCUSSION OF VITAMIN A STUDY

In this study a wide dosage range from 1 000 to 50 000 IU vitamin A per mouse was used, which resulted in a spectrum from no detectable embryotoxic effect, through teratogenic to embryolethal. With the exception of 2 experiments a dose of 10 000 IU was the maximum dose used in the definitive study and was not associated with any significant maternal toxicity.

In the absence of evidence of maternal toxicity obvious teratogenic

effects were observed in the offspring, which is in keeping with the concept of a direct effect of vitamin A on the embryo (see section 1.2).

The time of administration varied from day 7 to 17 of gestation which enabled testing the effects of the teratogen during the embryonic phase of development and during fetogenesis. Most studies were performed during an important period of brain development. Dosing on a single day was found to be adequate to produce a teratogenic effect.

The time of sacrifice chosen also varied. In many studies this was 1 or 2 days prior to term when substantial enzyme activity was present. This ensured a good chance of detecting small changes in enzyme activity. Fetuses were also sacrificed earlier in gestation, such as on day 12, to limit the possibility of repair masking embryotoxic effects. This, however, necessitated using the whole head because of the technical difficulty of removing the brain, and enzyme activity was substantially lower. Fetuses were not studied post-natally because of the likelihood of dams cannibalising abnormal fetuses.

The use of controls kept under identical conditions in each experiment accounted for variation in such parameters as fetal weight and AChE activity in different experiments.

A possible criticism may be that larger sample sizes might have been used. This, however, was not possible because assays were performed on fresh brain homogenates where test and control animals were only compared in the same experiment, limiting the number of brains that could be assayed. On reviewing the study it is improbable that larger sample sizes or repeat experiments would have improved precision substantially.

An obvious difference in severity and frequency of congenital defects from litter to litter as well as from offspring to offspring within the same litter was apparent. This phenomenon is well known in teratology and may depend on biochemical or morphological variations that are genetically determined (Wilson, 1977 : 49).

Resorptions

Resorption was a useful indicator of embryotoxicity. There was an increase in the incidence of resorptions with an increase in dose. Similarly dosing earlier in gestation e.g. on days 7, 8 or 9 was associated with a substantial increase in resorption rate. These findings are in agreement with those of other workers (Kalter and Warkany, 1961).

Intra-uterine deaths

With the range of doses and times of administration of vitamin A used in this study, it is interesting that embryotoxicity was manifest either as embryolethality resulting in resorption or early intra-uterine death, or malformations, without evidence of intra-uterine death arising later in gestation. This contrasts with the findings of the cyclophosphamide study with administration during the same period of gestation (see Chapter 7).

Morphological abnormalities

There was a dramatic increase in incidence of gross morphological abnormalities with increasing dose, jumping from 9,1% with 5 000 IU vitamin A on day 9 to 100% with 10 000 IU on day 9. The incidence with 10 000 IU administered on day 10 was lower at 42,3 to 54,5%. This indicates a very steep dose response curve for malformations induced by vitamin A in C3H mouse fetuses, which is to the left of that for embryolethality. The gross abnormalities are similar to those noted by others (Kalter and

Warkany, 1961; Kalter, 1968; Geelan, 1979), although the strikingly high incidence of exophthalmos with the characteristic abnormal shaped head (38%) and the high incidence of tail abnormalities (66%) have not been described by other workers and probably indicate a particular susceptibility of C3H mice with dosing on day 9 or 10 of gestation.

Fetal weight and brain weight

There was no consistent effect on these parameters compared with controls except with 10 000 IU on day 9 and 3 000 IU consecutively on days 8 and 9 when fetal weight,and in some experiments brain weight,were significantly reduced. This is compatible with a growth inhibitory effect of vitamin A. Of note is that this was only observed when teratogenic doses were used, nor was this apparent when dosing later on day 10 with the exception of one experiment (section 6.6.5) where a high dose of 15 000 on day 10 was used. This contrasts with cyclophosphamide which can cause a growth retarding effect when doses that are not teratogenic are used and is growth inhibitory when administered later in gestation (see Chapter 7). A growth retarding effect of vitamin A has been noted by other workers (Morriss and Steele, 1974; Morriss and Steele, 1977; Kochar, 1968).

Acetylcholinesterase

I have shown that vitamin A, independent of its embryotoxic effects, did not alter AChE activity.

A dose of 1 000 IU vitamin A administered on day 9 did not appear to be teratogenic: no abnormalities were noted in any of the parameters examined. A non-teratogenic dose was not associated with any change in AChE activity.

Studies with 5 000 IU on day 9 or day 10 were not associated with a consistent change in any of the parameters examined, although a teratogenic

effect was grossly apparent in 2 fetuses with exophthalmos. Examination of fetuses 48h after dosing reduced the possibility that repair of any CNS damage could have accounted for an absence of altered AChE activity.

The administration of 10 000 IU of vitamin A on day 10 was associated with a 42 - 54% incidence of gross malformations and an increase in brain AChE ($p<0,005$). The mechanism for the increase in brain AChE activity could be reasonably explained on the basis of relative sparing of cholinergic neurons from the CNS damage caused by the teratogen, i.e. that cholinergic neurons are relatively more resistant than other neuronal cells. This suggestion is in agreement with that of Adlard and Dobbing (1972) and Valcana and Timiras (1974) who noted increased brain AChE in rats exposed to X-irradiation. There is also histological evidence that radiation results in reduced numbers of granule, basket and stellate cells of the cerebellar cortex, but that cholinergic golgi cells are unaffected (Altman and Das, 1970; Altman and Anderson, 1971; Ebels, 1970). The findings of Langman and Welch (1967) that Swiss-Webster mice treated with vitamin A had reduced numbers and abnormal differentiation of neuroblasts in the cerebral cortex indicates the susceptibility of this population of cells. It is quite conceivable that the ontogenetically older cholinergic neurons are relatively unaffected.

An alternative explanation is that the increase in AChE activity could be a reflection of exuberant repair or growth of cholinergic neurons. The findings of Smith et al., (1978) of hyperplasia and overgrowth of neural tube tissue in rat fetuses exposed to vitamin A are consistent with this concept. Conceptually, however, exuberant repair is more acceptable than involking a teratogenic mechanism which directly stimulates cholinergic neurons. The latter might imply a growth stimulatory effect which is contrary to the reported growth inhibitory effect of vitamin A, although

no growth inhibition was evident with this regimen. The absence of an increase in fetal weight or brain total protein content argue against increased growth. Similarly, the absence of an increase in ChAT activity tends to point against exuberant repair.

The absence of an increase in brain total protein content in the face of higher AChE activity suggested that the greater enzyme activity was not merely the result of a general increase in brain total protein content.

Examination of the isoenzymes of AChE showed that the sum of the peak heights of the isoenzyme bands were greater for test brains ($p = 0,05$) which was consistent with the findings above. The predominant increase in enzyme activity was noted in band 5 ($p = 0,05$), although the median peak heights for bands 1 and 2 were also greater for tests, although not demonstrably.

Despite a higher incidence of gross morphological abnormalities with teratogenic doses of vitamin A, dosing earlier on days 8 and 9, when the fetal CNS is likely to be highly susceptible to teratogenic insults, was not associated with a significant change in brain AChE activity in whole brain, cortex or cerebellum. The explanation for this may have been a growth inhibitory effect of the vitamin as suggested by the lower fetal and brain weights. As noted in the cyclophosphamide experiments (see Chapter 7) this was associated with a lower AChE activity, but could have been offset by another teratogenic effect of vitamin A, which in previous experiments was associated with an increase in enzyme activity, the net result reflecting no significant change.

The absence of any change in AChE activity in cortex and cerebellum tended to exclude the possibility that changes in specific brain regions

in opposite directions accounted for the absence of an alteration in whole brain AChE.

As teratological effects are influenced by the time of exposure or developmental stage of an organ, this may explain the discrepancy in the above findings. (For example, even opposite effects have been observed by administration of the same dose of vitamin A one day apart. (Seller et al., (1979) noted an increased incidence of neural tube defects in curly-tailed mice when vitamin A was administered on day 8, but a decreased incidence when given on day 9). Perhaps a different mechanism operates with dosing on days 8 or 9 as opposed to day 10, the one associated with a change in enzyme activity and the other not. This exposes a definite potential weakness of a biochemical parameter such as AChE. Depending on the interaction of a number of factors such as growth inhibition, cell damage, relative sparing, repair, direct effects or different changes in different parts of an organ to increase or decrease the parameter examined, there may or may not be a net change. This is highlighted by the fact that when a more pronounced biochemical disturbance might have been anticipated with dosing on day 9 as opposed to day 10, none was found. This also illustrates that AChE alone is not a reliable marker of teratogenic injury induced by vitamin A, and emphasises the importance of considering all parameters when assessing a potential embryotoxic effect, including growth inhibition, malformations and embryolethality. Another potential limitation of AChE as a brain marker of teratogenic injury in the mouse fetus was revealed by the low AChE activity in the ontogenetically new cerebral cortex which would be expected to be the most susceptible to teratogenic insult.

The likelihood that the absence of any alteration in AChE activity in the above experiments was due to repair was diminished by the fact that no

change in enzyme activity could be demonstrated in fetuses examined 72h after dosing on day 12. However, repair appears to be a rapid process and could have been complete by this time (Langman et al., 1980). The absence of an increase in AChE activity in these 12 day fetuses suggests that the mechanism of embryotoxicity of vitamin A is not associated with a haemorrhagic process (contrast cyclophosphamide, Chapter 7).

Using the same dose of 10 000 IU later in gestation during the period of fetogenesis was not associated with any abnormality in any of the parameters studied i.e. it did not appear to be teratogenic when administered at this time and was not associated with a change in AChE activity. Larger doses of a teratogen are usually needed to produce embryotoxic effects later in gestation but I did not pursue studies during fetogenesis because of the somewhat disappointing results during the more sensitive period of organogenesis and the fact that large doses can result in maternal toxicity, which could confound interpretation of results.

Although it is conceivable that changes in post-natal brain AChE activity might occur after exposure to vitamin A in the absence of pre-natal changes, post-natal studies may be of limited value because mothers are known to cannibalise abnormal offspring (Palmer, 1978; Wilson, 1973).

6.9. CONCLUSIONS

It is concluded from this study that:

a) A dosage regimen of 10 000 IU vitamin A or less was not associated with significant maternal toxicity.

b) In vivo maternal administration of vitamin A caused a dose dependent

increase in resorption rate.

c) Dosing earlier in gestation resulted in a steep increase in the number of resorptions.

d) There was a steep dose response curve for malformations induced by vitamin A which increased from 9,1% to 100% by doubling the dose from 5 000 to 10 000 IU when administered on day 9 p.c. and from 0% to 54,5% when administered on day 10 p.c.

e) The high incidence of exophthalmos/abnormally shaped head and the high incidence of tail abnormalities indicated a particular sensitivity of C3H mice to these defects after exposure to vitamin A on day 9 or 10 of gestation.

f) Teratogenic doses of 10 000 IU vitamin A administered on day 8 or 9 of gestation, were associated with a small decrease in fetal weight which is compatible with a growth inhibitory effect.

g) Vitamin A, independent of its embryotoxic effects, did not alter AChE activity.

h) In the absence of evidence of a significant teratogenic effect of vitamin A, no change in brain AChE activity was demonstrable.

i) With the administration of an obviously teratogenic dose of vitamin A during the period of CNS development the presence or absence of a change in brain AChE activity depended on the time of administration.

j) Ten thousand IU vitamin A administrered on day 9 was associated with a 100% incidence of malformations ($p<0,001$) and a decrease in fetal weight (1 160mg compared with 1 250mg in controls, $p = 0,06$) and a de-

crease in brain weight (78,4mg compared with 82,7mg in controls, $p = 0,07$), but was not associated with a change in AChE in whole brain, cortex or cerebellum compared with controls).

k) The likelihood that the absence of any alteration in AChE referred to in (j) above could be accounted for by repair was diminished by the absence of any change in enzyme activity in fetuses examined 72h after dosing.

l) The administration of 10 000 IU of vitamin A on day 10 was associated with a 42% and 54% incidence of malformations ($p<0,01$) and a significant increase in brain AChE (3,20 compared with 2,99nmol/min/mg in controls in the first experiment, $p<0,005$, and 3,55 compared with 3,19nmol/min/mg in controls in the second experiment ($p<0,0005$).

m) The increase in brain AChE in (l) above was not associated with a demonstrable increase in fetal weight, brain total protein content or ChAT activity and was therefore probably not related to enhanced growth or exuberant repair of cholinergic neurons.

n) The administration of 10 000 IU vitamin A on day 10 was associated with a significantly higher AChE activity of band 5 and the sum of the activities of the isoenzyme zones ($p = 0,05$).

o) A dose of 10 000 IU administered later in gestation on day 13 p.c., was not associated with a significant effect on any of the parameters studies i.e. did not appear to be embryotoxic.

p) Brain AChE alone is not a reliable indicator of teratogenic injury induced by vitamin A in C3H mice.

CHAPTER 7

A STUDY OF THE EFFECTS OF IN VIVO MATERNAL ADMINISTRATION OF CYCLOPHOSPHAMIDE ON THE C3H MOUSE FETUS

7.1. INTRODUCTION

A discussion on cyclophosphamide as a teratogen, with particular reference to its effects on the developing CNS, has been given in section 1.3.

The aims of this study were:

i) To investigate the effects of cyclophosphamide when administered during embryonic development, including an important period of CNS development, on gross fetal parameters in C3H mice, including embryolethality, gross morphological abnormalities, fetal weight and brain weight;

ii) to compare these with the effects on brain AChE and its isoenzymes, and with brain ChAT;

iii) to compare the effects with those observed using vitamin A in the previous study, and further evaluate AChE as a potential brain biochemical marker of teratogenic injury;

iv) to elucidate the teratogenic mechanisms of cyclophosphamide with particular reference to the central nervous system.

7.2. MATERIALS AND METHODS

7.2.1. Experimental mice

Timed matings were performed with young healthy virgin female C3H mice aged 8 - 12 weeks weighing 19 - 23g, as described in section 2.4., and kept under standard experimental conditions (section 2.3). Two-hour matings were performed. Pregnant females were randomly divided into test and control groups.

7.2.2. Drug administration

Cyclophosphamide was diluted in sterile water and administered by subcutaneous (s.c.) injection. The doses used were 7,5-20mg/kg per mouse (see section 7.3.) An equal volume of distilled water was administered s.c. to controls.

7.2.3. Records of maternal and fetal parameters and statistical analysis

Maternal Parameters. The weight on the day of mating, day of dosing and the day of sacrifice and the physical state of each mouse after dosing and before sacrifice were recorded.

Fetal Parameters. Resorptions, intra-uterine deaths, gross morphological abnormalities, fetal weights, brain weights, brain AChE activity, and in some instances AChE isoenzymes and choline acetyltransferase activity were investigated.

Statistical Analysis. The incidence of resorptions, intra-uterine deaths and morphological abnormalities was compared with controls using the Chi-square two-tailed test. Fetal weights, brain weights and brain AChE activity were compared with controls using the Mann-Whitney U two-tailed test. Medians and 95% confidence limits were calculated from the Walsh averages, after Tukey (Steinijans and Diletti, 1983).

7.3. PRELIMINARY EXPERIMENTS

Guided by the experimental literature, as presented in section 1.3, the susceptibility of C3H mice to cyclophosphamide under their experimental conditions was determined. A suitable dosage range was found to be 7,5-20mg/kg which produced a spectrum of effects from growth inhibition alone at 7,5mg/kg to a high incidence of gross malformations and embryolethality at 20mg/kg. (These effects are evident in the experiments presented in section 7.4). The time of administration chosen was day 8 to 12 (single administration), because this falls within an important period of CNS de-

velopment, and includes the most sensitive period of exposure for producing murine malformations (Gibson and Becker, 1968). Other preliminary experiments were performed

a) to determine that the doses used were less than the minimal toxic dose for the mothers and

b) to determine whether cyclophosphamide per se affects AChE.

7.3.1. Study to determine whether the doses of cyclophosphamide used were below the minimal toxic dose level (M.T.D.) for the adult C3H females used

This experiment was performed as described for vitamin A in section 6.3.9. Cyclophsophamide, 20mg/kg s.c. was administered to 12 animals on the first day. The mice were observed for signs of toxicity and were reweighed nine days after dosing. (Large numbers of mice were not used as this was thought to be wasteful and unnecessary).

Results and conclusion

No signs of toxicity or deaths were noted. Table 7.1. gives the mouse weights on day 0 and day 9 and the difference in weight. In 8 mice the weight was unchanged, in 3 there was a gain in weight of 0,5 to 1g and in 2 a loss of weight of 1g.

Although the numbers are too small for adequate statistical analysis it is apparent there was no obvious toxic effect on adult C3H mice. (A dose of 20mg/kg cyclophosphamide was the maximum dose used in this study).

7.3.2. Experiment to determine whether cyclophosphamide, independent of its teratogenic effects, alters brain acetylcholinesterase activity

Cyclophosphamide, 12,5mg/kg, which was found in preliminary experiments to be teratogenic when administered on day 10 p.c., was administered on day 17 p.c. and fetuses sacrificed the following day, i.e. on day 18 p.c. Day 17 was chosen because this is late in the period of fetogenesis when susceptibility to teratogenic effects is unlikely. Sacrifice 24h later

TABLE 7.1.

MOUSE WEIGHTS ON DAY OF ADMINISTRATION OF 20 mg/kg CYCLOPHOSPHAMIDE (DAY 0) AND 9 DAYS LATER

WEIGHT IN GRAMS		
DAY 0	DAY 9	WEIGHT CHANGE
23	23	0
23	23,5	+0.5
22	22	0
23	22	-1
23	23	0
23	24	+1
23	23	0
22	22,5	+0.5
23	23	0
23	22	-1
23	23	0
22	22	0

ensured adequate time for the drug to have entered the fetal CNS and produce any direct effects.

Results are shown in Tables 7.2T and 7.2C (original data, pp. 406 and 407).
A summary of results is given in Table 7.3.
There was no significant difference in AChE activity between tests and controls, nor in fetal or brain weight.

Conclusion

The absence of any difference in AChE between the group treated with 12,5mg/kg cyclophosphamide compared with controls suggests that cyclophosphamide, independent of its embryotoxic effects, does not alter fetal brain AChE activity.

7.4. RESULTS OF CYCLOPHOSPHAMIDE STUDY

Tables of original data are presented at the end of the thesis.
Summary Tables are given with the text.
Table 7.4. shows the doses used, the day of administration, the day of sacrifice and the specific assay performed.

7.4.1. Cyclophosphamide, 20mg/kg per mouse, administered on day 10 p.c. (sacrifice day 19 p.c.)

See Tables 7.5T and 7.5C for original data (pp. 408 and 409).
Summary of results in Table 7.6.
Note: There were no signs of maternal toxicity in this or any of the subsequent experiments with cyclophosphamide in this chapter. Weight gain appeared similar after dosing to that of controls but was not statistically compared because of the small numbers in each group.

There was a 78% incidence of resorptions (43 of 55 conceptions) in the

T A B L E 7.3.

SUMMARY OF RESULTS OF 12,5mg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 17 p.c. (SACRIFICE DAY 18 p.c.)

	TEST	CONTROL	p
Total Conceptions	20	22	-
Resorptions (%)	5	9,1	NS
Intra-uterine Deaths (%)	0	9,1	NS
Abnormalities (%)	0	5,5	NS
Median Fetal Weight (g)	1,076 (1,028 - 1,121)	1,068 (1,025 - 1,104)	NS
Median Brain Weight (mg)	73,10 (65,85 -75 ,55)	74,10 (71,75 - 76,25)	NS
Median AChE Activity nmol/min/mg	2,328 (2,196 - 2,502)	2,458 (2,262 - 2,578)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

TABLE 7.4.

CYCLOPHOSPHAMIDE STUDY : SUMMARY OF EXPERIMENTS PERFORMED SHOWING DOSE OF CYCLOPHOSPHAMIDE, DAY OF ADMINISTRATION, DAY OF SACRIFICE, AND THE SPECIFIC ASSAY PERFORMED

Dose of cyclophosphamide mg/kg	Day of administration	Day of sacrifice	Assay
20	10	19	Whole brain AChE
12,5	10	19	" " "
12,5	10	19	" " "
12,5	10	18	" " "
15	10	18	" " "
7,5	10	19	" " "
12,5	8½	18	-
12,5	10	14	Whole head AChE
20	12	13	" " "
20	10	12	" " "
20	10	12	" " "
20	10	12	Whole head haemoglobin
20	10	14	" " "
15	10	18	Whole brain ChAT
20	10	12	Whole head ChAT
12,5	10	18	Brain isoenzymes

T A B L E 7.6.

SUMMARY OF RESULTS OF 20mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 19 p.c.)

	TEST	CONTROL	p
Total Conceptions	55	21	-
Resorptions (%)	78	0	<0,001
Intra-uterine Deaths (%)	2	4,8	NS
Abnormalities (%)	100	0	<0,001
Median Fetal Weight (g)	0,89 (0,86 - 0,94)	1,285 (1,19 - 1,35)	<0,00006
Median Brain Weight (mg)	62,3 (60,7 - 64,0)	84,78 (81,6 - 87,5)	<0,00006
Median AChE Activity nmol/min/mg	2,98 (2,89 - 3,07)	3,28 (3,2 - 3,43)	<0,0014

Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

treated group compared with 0% in controls, and 1 intra-uterine death in each of the test and control groups. All of the 11 viable fetuses were abnormal in the treated group. (There were no gross abnormalities in the control group). Morphological abnormalities included prominent eyes, frank exophthalmos, and heads which were shortened anteroposteriorly with hypoplastic faces. Test fetuses were obviously smaller than controls.

The median fetal weight for the test group (890mg) was significantly lower than for the control group (1 285mg; $p<0,00006$), as was the median brain weight of treated fetuses (62,3mg) compared with 84,8mg for the control group ($p<0,00006$).

The median brain AChE activity of test animals, 2,98nmol/min/mg was significantly lower than that for controls (3,28nmol/min/mg; $p = 0,0014$).

Discussion

The 78% resorption rate demonstrated that 20mg/kg cyclophosphamide administered on day 10 to C3H mice was embryolethal. The fact that there were 44 resorptions and only 1 IUD in a near-term fetus suggested that cyclophosphamide at this dose exerted its main embryolethal effect in C3H mice early after administration. (The term resorption was used here because the $\pm$ 2mm necrotic fetal remnants appeared to have largely resorbed).

The high incidence of resorptions and the 100% incidence of gross abnormalities demonstrated that a dose of 20mg/kg was teratogenic.

The decrease in fetal weight and brain weight were in keeping with the growth inhibitory effect of cyclophosphamide (see section 1.3.) The

significant decrease in brain AChE was also in keeping with growth inhibition. (Because there is an increase in fetal weight, brain weight and brain AChE with increasing age, a growth inhibitory effect would be expected to be associated with a decrease in these parameters (see section 3.3). Another superimposed mechanism, such as a teratogenic one affecting AChE, could not be excluded, however.

7.4.2. Cyclophosphamide, 12,5mg/kg per mouse, administered on day 10 p.c. (sacrifice day 18 and 19 p.c.)

Three experiments were performed.

See Tables 7.7., 7.9., 7.11 for original data (pp. 410-416).

Summaries of results are presented in Tables 7.8., 7.10. and 7.12.

Overall for the 3 experiments there was an 11,1% incidence of resorptions compared with 6,6% in controls, which was not significant, an 11,1% incidence of intra-uterine deaths compared with 5,3% in controls ($p<0,025$) and an 11,3% incidence of gross morphological abnormalities compared with 1,9% in controls ($p<0,025$). Treated fetuses were obviously smaller than controls.

Fetal weights were lower in the test groups compared with controls in all 3 experiments ($p<0,00006$). Median fetal weight for test animals in the first experiment was 986mg compared with 1 268mg for controls.

Brain weights were also lower in the test groups compared with controls in all 3 experiments ($p<0,00006$). In the first experiment the median weight for tests was 68,15mg and for controls 84,15mg.

Brain AChE activity was significantly lower for tests than for controls in all 3 experiments ($p<0,00006$). In the first experiment median AChE for tests was 2,78 and for controls 3,158nmol/min/mg.

The lower fetal weight, brain weight and brain AChE of the treated group compared with controls in the first experiment is demonstrated graphically in Fig. 7.1.

T A B L E 7.8.

SUMMARY OF RESULTS OF 12,5mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 19 p.c.) (FIRST EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	33	16	-
Resorptions (%)	21,2	0	0,1-0,05
Intra-uterine Deaths (%)	9,0	0	NS
Abnormalities (%)	21,7	0	0,1-0,05
Median Fetal Weight (g)	0,986 (0,94 - 1,03)	1,27 (1,19 - 1,33)	<0,00006
Median Brain Weight (mg)	68,15 (66,1 - 70,4)	84,15 (81,6 - 86,0)	<0,00006
Median AChE Activity nmol/min/mg	2,78 (2,63 - 2,91)	3,16 (3,06 - 3,26)	<0,00006

Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

T A B L E 7.10.

SUMMARY OF RESULTS OF 12,5mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 19 p.c.) (SECOND EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	20	23	-
Resorptions (%)	0	13,0	NS
Intra-uterine Deaths (%)	10	4,3	NS
Abnormalities (%)	5,5	5,3	NS
Median Fetal Weight (g)	1,106 (1,06 - 1,15)	1,320 (1,26 - 1,38)	<0,00006
Median Brain Weight (mg)	74,45 (72,1 - 76,7)	86,78 (83,6 - 88,6)	<0,00006
Median AChE Activity nmol/min/mg	2,928 (2,84 - 3,0)	3,263 (3,17 - 3,35)	<0,00006

Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

T A B L E 7.12.

SUMMARY OF RESULTS OF 12,5mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 18 p.c.) (THIRD EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	28	22	-
Resorptions (%)	7,14	4,5	NS
Intra-uterine Deaths (%)	14,3	9,0	NS
Abnormalities (%)	4,3	0	NS
Median Fetal Weight (g)	0,836 (0,8 - 0,88)	1,028 (1,0 - 1,05)	< 0,00006
Median Brain Weight (mg)	57,7 (56,0 - 59,3)	70,3 (68,9 - 71,5)	< 0,00006
Median AChE Activity nmol/min/mg	2,627 (2,52 - 2,71)	2,937 (2,87 - 3,0)	< 0,00006

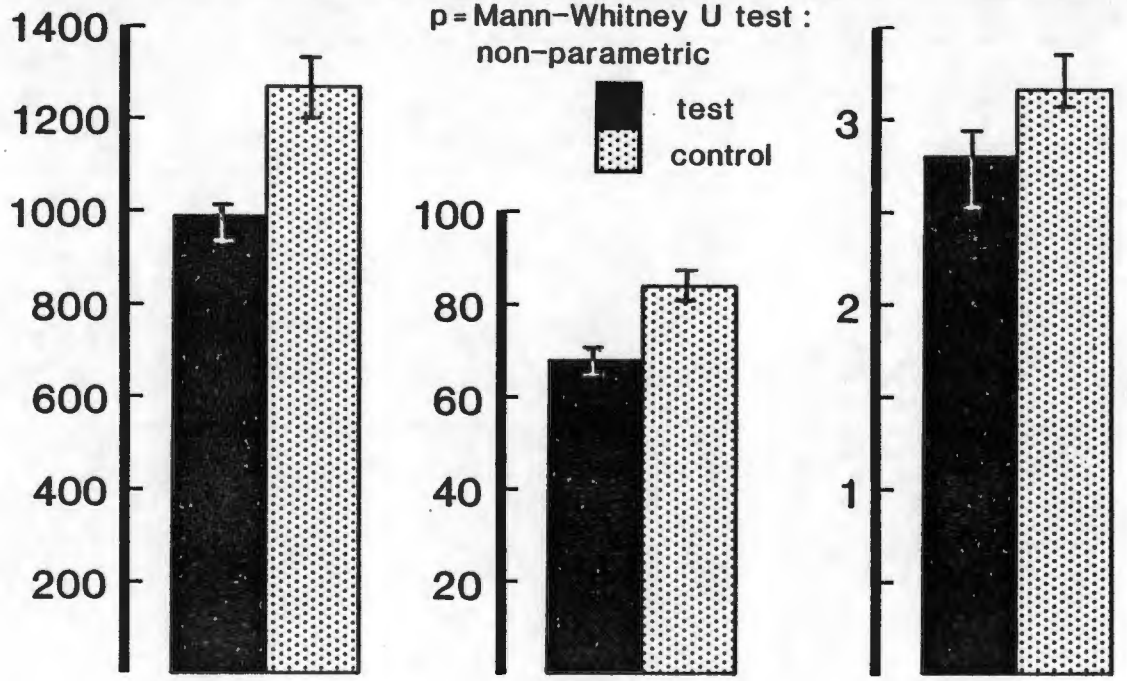
Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

Fig. 7.1.

THE EFFECT OF CYCLOPHOSPHAMIDE (12.5mg/kg D10) ON FETAL & BRAIN WEIGHT & AChE ACTIVITY

	MEDIAN FETAL WEIGHT (mg)	MEDIAN BRAIN WEIGHT (mg)	MEDIAN AChE ACTIVITY (nmol/min/mg)
p	<0.00006	<0.00006	<0.00006



Discussion

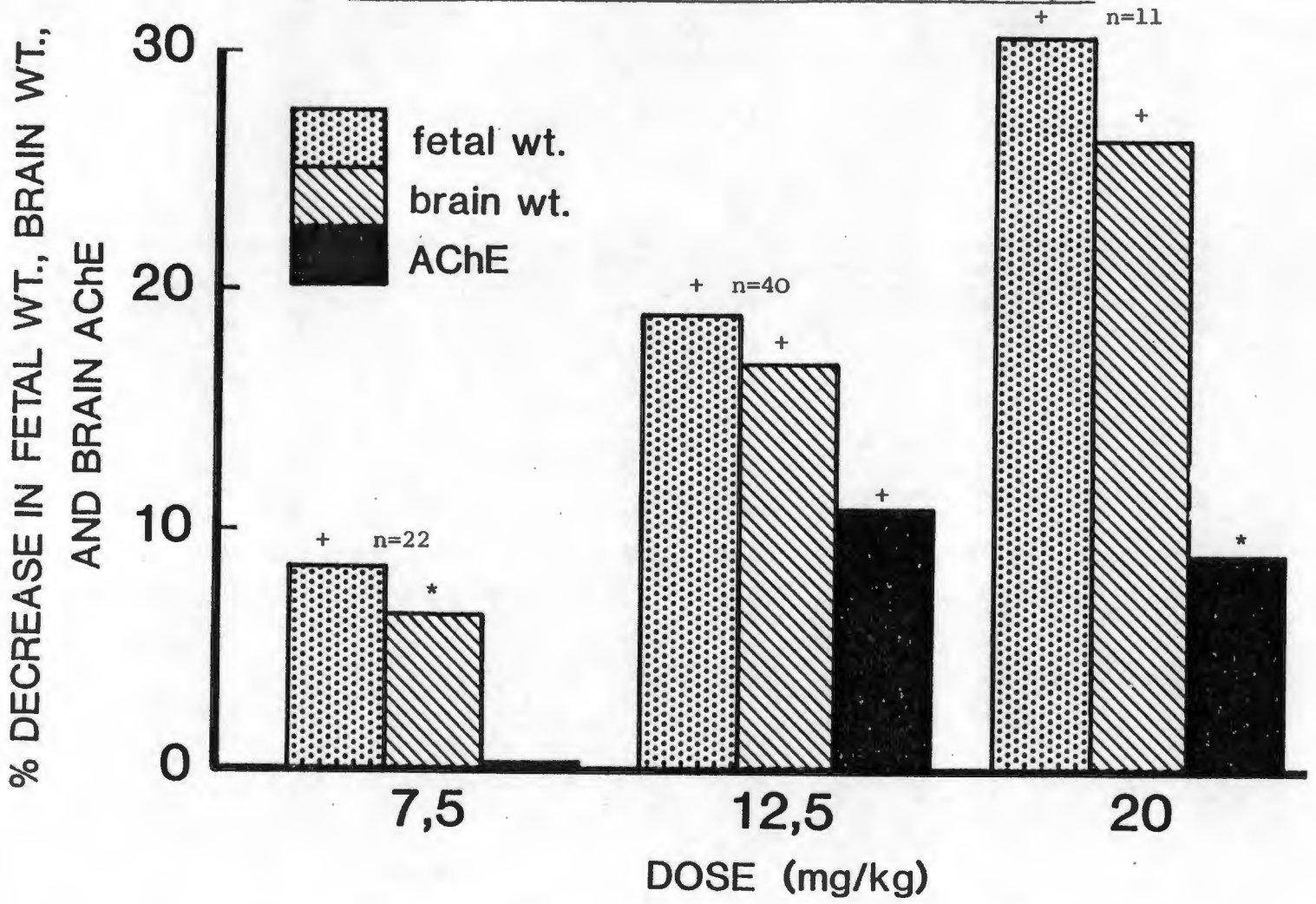
A much lower incidence of resorptions (11,1% overall in the treated group versus 6,6% in the controls, $p<0,1$) was apparent compared with 78% at a dose of 20mg/kg cyclophosphamide administered on day 10 p.c., confirming a smaller embryolethal effect with the lower dose. There was also an overall 11,1% incidence of IUD's compared with 5,4% in controls ($p<0,025$) which was further evidence for a significant but small embryolethal effect of 12,5mg cyclophosphamide administered on day 10 p.c.

The 11,3% incidence of gross morphological abnormalities compared with 1,9% in controls ($p<0,025$) was in keeping with a teratogenic effect, but 12,5mg/kg was obviously less potent than 20mg/kg. Of note was that a significant incidence of gross morphological abnormalities was only apparent in the first experiment. Although gross abnormalities are a relatively crude indicator of teratogenic effects this would be in keeping with the probability that 12,5mg/kg is at the lower end of the teratogenic dose-response curve for cyclophosphamide in the C3H mice used, where abnormalities in only a few fetuses within a limited number of litters would be expected.

Cyclophosphamide 12,5mg/kg on day 10 resulted in a 16 - 22% decrease in fetal weight, a 14 - 19% decrease in brain weight and a 10 - 12% decrease in brain AChE activity compared with controls in the 3 experiments, which were in keeping with the growth inhibitory effect of the drug. Of interest, with a larger dose of 20mg/kg cyclophosphamide administered on day 10, the decrease in brain AChE activity was marginally smaller at 9,15%, despite a greater decrease in fetal weight of 30,7% and a greater decrease in brain weight of 26,5%. The percentage decrease in fetal weight, brain weight and brain AChE with increasing doses of cyclophosphamide is shown in Table 7.13 and Fig. 7.2.

Fig. 7.2.

THE PERCENTAGE DECREASE IN FETAL WEIGHT, BRAIN WEIGHT AND ACETYLCHOLINESTERASE WITH INCREASING DOSES OF CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c.



Comparison with parameters for controls, n=35, 22, 21, respectively.

+ $p<0,00006$

* $p<0,001$

T A B L E 7.13.

THE PERCENTAGE DECREASE IN FETAL WEIGHT, BRAIN WEIGHT AND BRAIN ACETYLCHOLINESTERASE WITH INCREASING DOSES OF CYCLOPHOSPHAMIDE

Dose (mg/kg)	% decrease in fetal wt.	% decrease in brain wt.	% decrease in AChE
7,5	8,5	6,5	0
12,5	19,03	17,03	11,0
20,0	30,7	26,5	9,15

It is evident that the percentage decrease in fetal and brain weight was proportional to the dose of cyclophosphamide administered, but this was not the case with brain AChE activity. This may suggest that the decrease in brain AChE was not entirely explicable on the basis of the growth inhibitory action of cyclophosphamide, although the percentage decrease in AChE need not parallel the decrease in fetal and brain weight.

An experiment was next performed with control fetuses sacrificed on days 17 and 18 p.c., and cyclophosphamide-treated fetuses sacrificed on day 18 p.c., in an attempt to compare brain AChE of treated with control fetuses of similar weight.

7.4.3. Cyclophosphamide, 15mg/kg, administered on day 10 p.c. (sacrifice day 18 p.c.) Controls sacrificed on days 17 and 18 p.c.

See Tables 7.14T and 7.14C for original data (pp. 417-421).

Summary of results in Tables 7.15 and 7.16.

TABLE 7.15.

SUMMARY OF RESULTS OF 15mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICED ON DAY 18 p.c. AND CONTROLS SACRIFICED ON DAY 17 p.c.

	TEST	CONTROL	p
Total Conceptions	46	28	-
Resorptions (%)	43,5	7,1	<0,01
Intra-uterine Deaths (%)	19,6	3,6	<0,02
Abnormalities (%)	6,0	0	NS
Median Fetal Weight (g)	0,796 (0,77-0,83)	0,681 (0,65-0,71)	<0,001
Median Brain Weight (mg)	57,95 (56,1-59,9)	54,60 (52,3-56,4)	<0,01
Median AChE Activity nmol/min/mg	2,382 (2,28-2,48)	1,882 (1,81-1,97)	<0,001

Figures in brackets are the 95% confidence range

T A B L E 7. 16.

SUMMARY OF RESULTS OF 15mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 18 p.c. AND CONTROLS SACRIFICED DAY 18 p.c.)

	TEST	CONTROL	p
Total Conceptions	46	20	-
Resorptions (%)	43,5	10	< 0,01
Intra-uterine Deaths (%)	19,6	0	< 0,05
Abnormalities (%)	6,0	0	NS
Median Fetal Weight (g)	0,796 (0,77-0,83)	0,996 (0,96-1,03)	<0,002
Median Brain Weight (mg)	57,95 (56,1-59,9)	67,35 (64,9-69,4)	<0,002
Median AChE Activity nmol/min/mg	2,382 (2,28-2,48)	2,425 (2,37-2,51)	NS

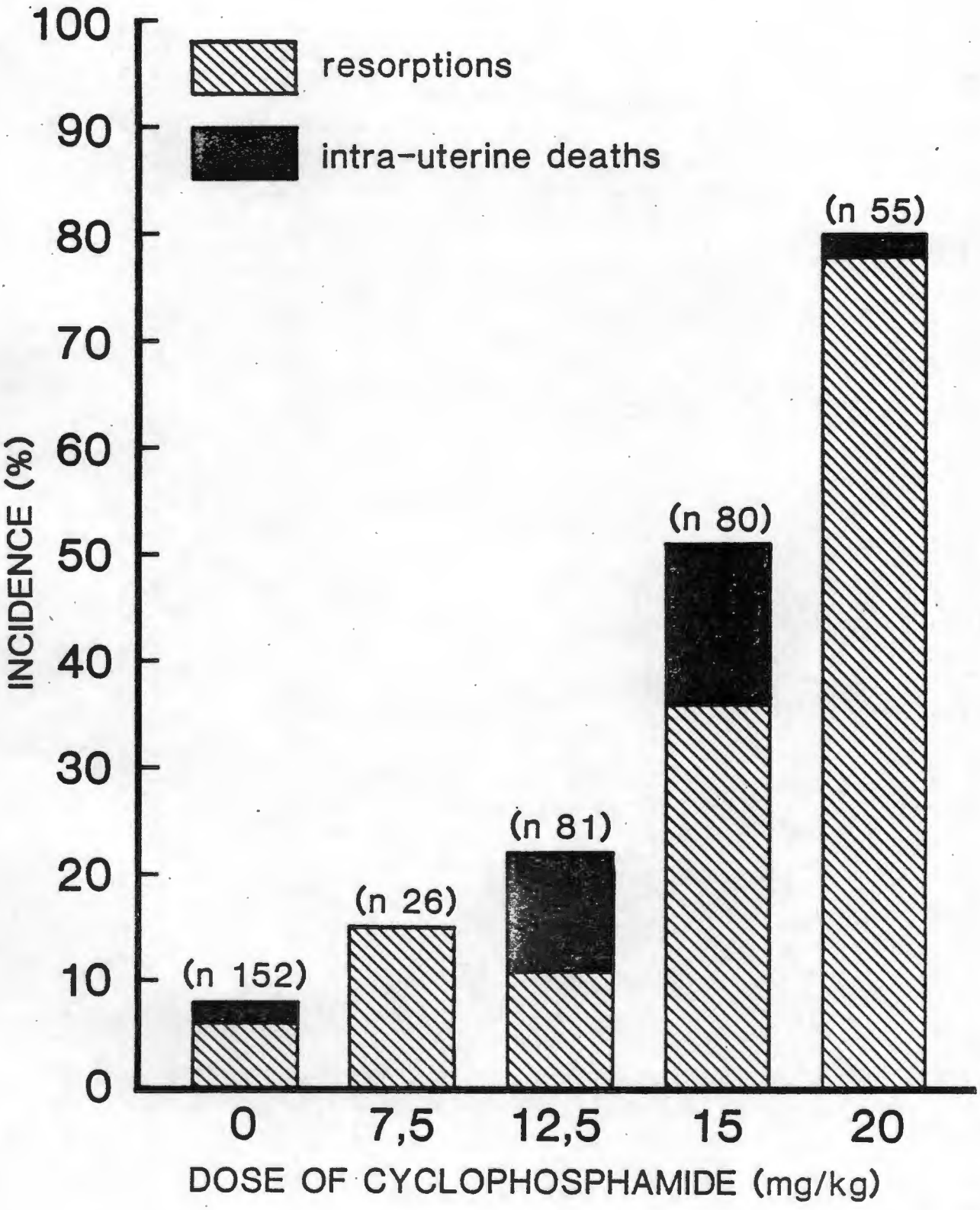
Figures in brackets are the 95% confidence range

There was a 43,5% incidence of resorptions in the treated group compared with 7,1% in controls ($p<0,01$). As expected the incidence of resorptions was higher than dosing with 12,5mg/kg (12,2%) and lower than dosing with 20mg/kg (78%) (see Fig. 7.3).

There was a 19,6% incidence of intra-uterine deaths compared with 3,6% in controls. This was higher than the 11,1% incidence with 12,5mg/kg cyclophosphamide. (The low incidence of 2% IUD's with 20mg/kg (section 7.4.1.) was probably accounted for by the high resorption rate (with a small number of remaining viable fetuses) demonstrating an earlier embryotoxic effect with this higher dose.

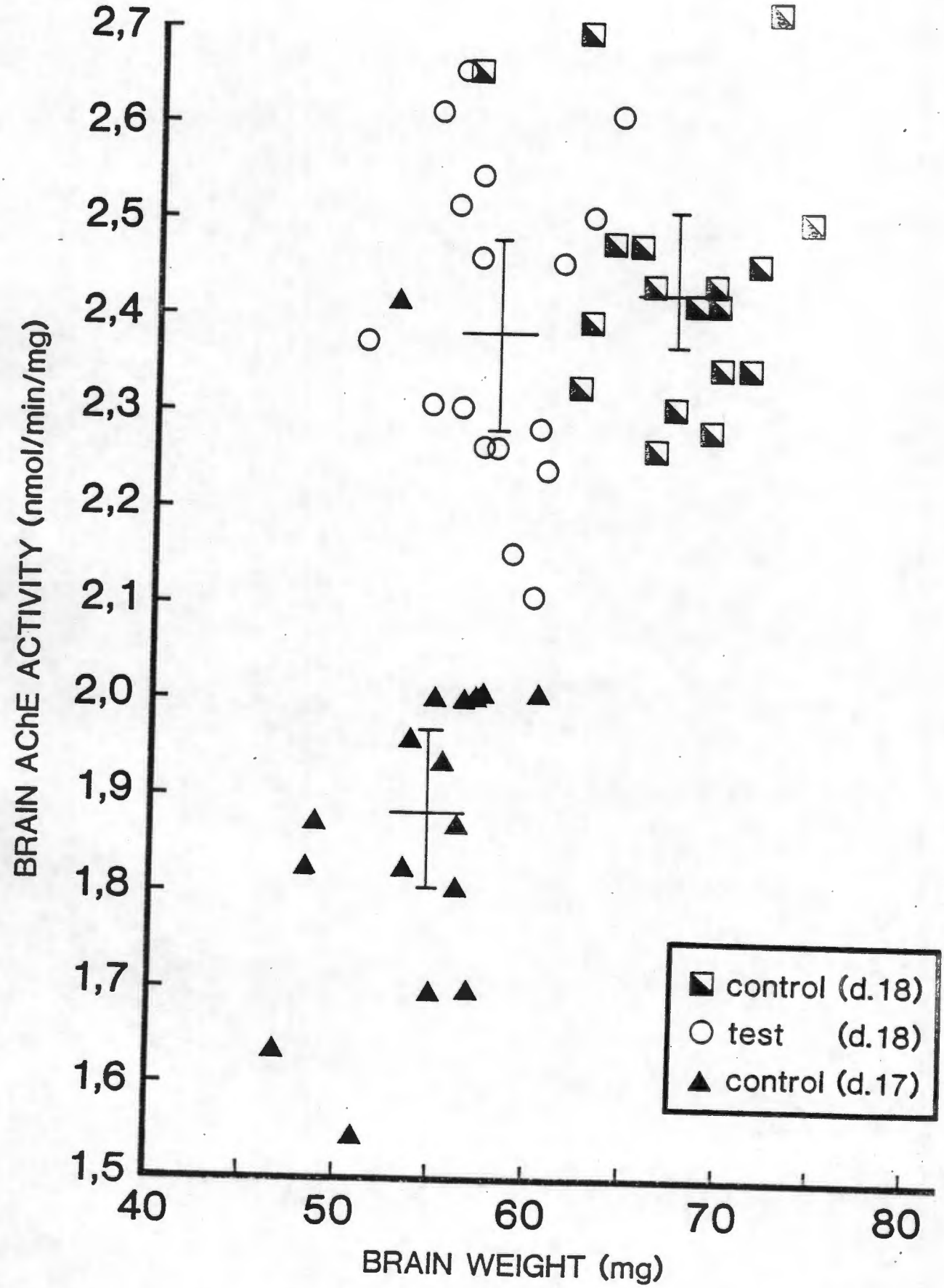
One fetus in the treated group had exophthalmos. There were no abnormalities in the control group. There was a lower fetal weight in the test group (median fetal weight 0,796g) compared with controls of the same age, i.e. 18 days p.c. (median weight 0,996g, $p<0,002$). There was also a significantly lower brain weight in the treated group (median 57,95mg) compared with controls of the same age (median 67,35mg, $p<0,002$). Although the median brain AChE activity in the treated group was lower than in controls of the same age (2,382 compared with 2,425nmol/min/mg respectively) this was not significant. However, brain AChE of 18 day treated fetuses was highly significantly greater than 17 day controls (median brain AChE 2,382 and 1,882nmol/min/mg respectively, ($p<0,001$) although median brain weight was only marginally higher in the 18 day treated group (median 57,95mg compared with 54,6mg, $p = 0,01$). Figure 7.4. shows the relationship of brain AChE activity to brain weight in the 17-day and 18-day controls and the 18-day cyclophosphamide-treated fetuses. The higher brain AChE activity of 18-day treated fetuses is apparent compared with the 17-day control fetuses of similar brain weight. For a given brain weight, therefore, cyclophosphamide-treated

Fig. 7.3.



THE RELATIONSHIP BETWEEN THE DOSE OF CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. AND THE INCIDENCE OF RESORPTIONS AND INTRA UTERINE DEATHS

Fig. 7.4.



THE RELATIONSHIP OF BRAIN AChE ACTIVITY TO BRAIN WEIGHT IN 17 AND 18 DAY UNTREATED FETUSES AND 18 DAY FETUSES EXPOSED TO 15mg/kg CYCLOPHOSPHAMIDE ON DAY 10 p.c.

Error bars are 95% confidence limits

fetuses have a higher AChE activity than controls, and one is tempted to speculate that, despite the effect of the teratogen to retard growth or development, and to therefore be associated with a decrease in AChE, this appears to have been offset by an independent effect of the teratogen to increase the enzyme. However, fetal weights (which would have been a more reliable index of growth inhibition) of the two groups were not comparable, the 18-day treated fetuses weighing significantly more than controls, which may have accounted for the difference in enzyme activity. Unfortunately it was apparent that to try and resolve the issue large numbers of fetuses would have been needed to enable comparison of adequate samples of similar weight.

7.4.4. Cyclophosphamide, 7,5mg/kg per mouse, administered on day 10 p.c. (sacrifice day 19 p.c.)

See Tables 7.17T and 7.17C for original data (pp. 422 and 423).

Summary of results in Table 7.18.

This experiment was performed to determine the effect of a lower dose of cyclophosphamide.

There were 4 resorptions in the test group (15,4%), 3 of which were in one animal, compared with 1 resorption in the controls. There were no intra-uterine deaths in the test group but 2 in the control group (8,0%). No gross morphological abnormalities were observed. Fetal and brain weights were significantly lower in the test group ($p<0,00006$ and $p = 0,0006$, respectively), but there was no meaningful difference in brain AChE activity between test and controls (median AChE values 3,304 and 3,296nmol/min/mg, respectively).

Discussion

The incidence of resorptions of 15,4% was higher than that with 12,5mg cyclophosphamide administered on day 10. However, three of the four

T A B L E 7.18.

SUMMARY OF RESULTS OF 7,5mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 19 p.c.)

	TEST	CONTROL	p
Total Conceptions	26	25	-
Resorptions (%)	15,4	4,0	< 0,05
Intra-uterine Deaths (%)	0	8,0	NS
Abnormalities (%)	0	0	NS
Median Fetal Weight (g)	1,24 (1,19 - 1,27)	1,35 (1,32 - 1,39)	< 0,00006
Median Brain Weight (mg)	80,5 (78,9 - 82,2)	86,1 (83,1 - 88,1)	0,0006
Median AChE Activity nmol/min/mg	3,304 (3,18 - 3,39)	3,296 (3,18 - 3,38)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

resorptions were in the same litter and probably either represent an unusually susceptible litter or a chance high spontaneous resorption rate. (The one resorption in the remaining 23 conceptions gives a resorption rate of 4,7%). The two IUD's in the control group are explicable on the basis of spontaneous deaths.

The significantly lower fetal and brain weights are again in keeping with the growth retarding effects of cyclophosphamide. Note, however, the proportionately smaller decrease in weight compared with higher doses (see Fig. 7.2). The lack of any significant difference in brain AChE may have been due to the fact that the mild growth inhibitory effect (there was only an 8,5% decrease in fetal weight and a 6,5% decrease in brain weight compared with controls) may not have been large enough to be associated with a decrease in brain AChE. Alternatively, although 7,5mg/kg is a growth inhibitory dose, the absence of morphological abnormalities may suggest the absence of other embryotoxic effects.

7.4.5. Cyclophosphamide, 12,5mg/kg per mouse, administered on day 8½ p.c. (sacrifice day 18)

See Table 7.19. for original data (p. 424).

This experiment was performed to compare the effects of the teratogen when administered earlier in gestation.

Three pregnant C3H females were used. Although they appeared healthy at the time of sacrifice there were 18 resorptions and only 1 live fetus which appeared grossly normal. (Controls were not sacrificed).

Discussion

Administering 12,5mg/kg cyclophosphamide on day 8½ as opposed to day 10 p.c. was highly embryolethal (94,7% incidence of resorptions), demonstrating

the considerably greater sensitivity of the embryo earlier in development.

Finding a single live, grossly normal fetus demonstrates the variability in susceptibility to a teratogen within the same litter.

Further Experiments

Because (i) repair of any CNS damage could have been complete by the usual time of sacrifice of day 18 or 19 p.c. and therefore mask biochemical changes, and (ii) inhibition of growth appeared to be marked by day 19 and was associated with a decrease in AChE activity at doses of 12,5 and 20mg/kg cyclophosphamide, fetuses were examined earlier (days 11 to 14 p.c.) in the following experiments. It was anticipated that examining fetuses soon after administration of the teratogen would minimise growth inhibitory effects, and repair, and therefore permit assessment of biochemical changes independently of the above influences.

Note:

a) The small size of the fetuses and the early stage of development made assessment of gross morphological abnormalities difficult.

b) The whole head (which comprises mainly brain at this early stage of gestation) was used for AChE assay.

7.4.6. Cyclophosphamide, 12,5mg/kg per mouse, administered on day 10 p.c. (sacrifice day 14 p.c.)

See Tables 7.20T and 7.20C for original data (pp. 425 and 426).

Summary of results in Table 7.21.

There was no difference in the incidence of resorptions or intrauterine deaths. As with sacrifice on day 19, the median fetal weight and brain weight of treated animals of 135mg and 51,1mg, respectively, were lower than for controls (169mg and 63,7mg, respectively, $p<0,00006$). However, no difference could be shown between whole head AChE activity of

T A B L E 7.21.

SUMMARY OF RESULTS OF 12,5mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 14 p.c.)

	TEST	CONTROL	p
Total Conceptions	22	21	-
Resorptions (%)	9,1	4,5	NS
Intra-uterine Deaths (%)	0	0	-
Abnormalities (%)	-	-	-
Median Fetal Weight (g)	0,135 (0,125 - 0,148)	0,169 (0,161 - 0,178)	<0,00006
Median Head Weight (mg)	51,1 (44,4 - 57,8)	63,65 (60,4 - 66,5)	<0,00006
Median AChE Activity nmol/min/mg	1,012 (0,96 - 1,07)	1,044 (1,0 - 1,1)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

test and control animals.

Discussion

The significant decrease in fetal weights and head weights in the treated group ($p<0,00006$) confirm an early growth inhibitory effect of cyclophosphamide. The absence of a difference in AChE (when a decrease in AChE was found with the same dose of cyclophosphamide but sacrifice on day 19) is interesting. The relevance of this became apparent after results from subsequent experiments were examined and will be discussed later.

Because of the possibility that repair could have been complete 4 days after dosing with cyclophosphamide (see discussion in section 1.3.), sacrifice was performed earlier in the ensuing experiments.

7.4.7. Cyclophosphamide, 20mg/kg per mouse, administered on day 12 p.c. (Sacrifice day 13 p.c.)

In an attempt to avoid working with fetuses that were too small, but which were sacrificed within a day or 2 of dosing, dosing on day 12 was used in this experiment.

See Tables 7.22T and 7.22C for original data (pp. 427 and 428).

Summary of results in Table 7.23.

There was no significant difference in fetal weight, head weight or AChE.

Discussion

The fact that there was not the usual decrease in fetal and head weight in the treated group could be explicable on the basis that a growth inhibitory effect was not significantly manifested as early as 24h after dosing. Alternatively, dosing later in gestation on day 12 as opposed to day 10, might be anticipated to be less embryotoxic and to not have as a pronounced a growth retarding effect.

TABLE 7.23.

SUMMARY OF RESULTS OF 20mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 12 p.c. (SACRIFICE DAY 13 p.c.)

	TEST	CONTROL	p
Total Conceptions	22	20	-
Resorptions (%)	4,5	10	NS
Intra-uterine Deaths (%)	0	0	-
Abnormalities (%)	0	0	-
Median Fetal Weight (g)	0,113 (0,109 - 0,118)	0,117 (0,110 - 0,124)	NS
Median Head Weight (mg)	45,75 (43,3 - 48,2)	49,45 (46,3 - 53,1)	NS
Median AChE Activity nmol/min/mg	0,805 (0,78 - 0,82)	0,816 (0,78 - 0,85)	NS

Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

7.4.8. Cyclophosphamide, 20mg/kg per mouse, administered on day 10 p.c. (Sacrifice day 12 p.c.)

Because of the absence of apparent embryotoxic effects with dosing on day 12 p.c., mice were dosed earlier on day 10 in this experiment.

See Tables 7.24T and 7.24C for original data (pp. 429-431).

Summary of results in Table 7.25.

The median fetal weight and brain weight of treated animals of 418mg and 15,5mg, respectively, were lower than for controls (714mg and 25,6mg, respectively, $p<0,00006$). A greater head AChE activity of 1,2nmol/min/mg in the test fetuses was apparent compared with controls (1,1nmol/min/mg, $p = 0,02$). Although 20mg/kg cyclophosphamide administered on day 10 was shown to produce a high resorption rate in an earlier experiment, treated fetuses were still viable as assessed by the presence of a regular heartbeat. The embryolethal effect of cyclophosphamide must therefore be manifest after day 12 (presumably shortly after day 12 to account for the high resorption rate in section 7.4.1).

Because of the unusual finding of a trend for a higher AChE activity in treated fetuses in the face of obvious growth retardation this experiment was repeated.

See Tables 7.26T and 7.26C (pp. 432 and 433).

Summary of results in Table 7.28.

Again an obviously lower fetal and head weight was apparent in the treated group ($p<0,00006$), and were associated with a greater head AChE activity of 1,24nmol/min/mg compared with controls (1,14nmol/min/mg, $p<0,001$).

Some control fetuses were sacrificed on day 11 p.c. to compare enzyme activity with day 12 p.c. (see Tables 7.29. and 7.30 , pp. 236 and 237).

T A B L E 7.25.

SUMMARY OF RESULTS OF 20mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 12 p.c.) (FIRST EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	32	21	-
Resorptions (%)	12,5	4,8	NS
Intra-uterine Deaths (%)	6,3	0	NS
Abnormalities (%)	15,4	0	0.1-0.05
Median Fetal Weight (g)	0,418 (0,38 - 0,45)	0,714 (0,66 - 0,77)	<0,00006
Median Head Weight (mg)	15,5 (13,9 - 17,0)	25,55 (23,2 - 27,8)	<0,00006
Median AChE Activity nmol/min/mg	1,175 (1,14 - 1,24)	1,11 (1,08 - 1,16)	0,02

Figures in brackets are the 95% confidence range
For statistical tests used see section 7.2.3.

T A B L E 7.28.

SUMMARY OF RESULTS OF 20mg/kg CYCLOPHOSPHAMIDE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 12 p.c.) (SECOND EXPERIMENT)

	TEST	CONTROL	p
Total Conceptions	22	26	-
Resorptions (%)	4,5	11,5	NS
Intra-uterine Deaths (%)	4,5	3,8	NS
Abnormalities (%)	15	0	0.1-0.05
Median Fetal Weight (g)	0,494 (45,95 - 52,3)	0,678 (63,5 - 71,7)	<0,00006
Median Head Weight (mg)	11,93 (10,2 - 13,85)	18,7 (16,5 - 21,0)	<0,00006
Median AChE Activity nmol/min/mg	1,24 (1,207 - 1,273)	1,142 (1,109 - 1,175)	<0,001

Figures in brackets are the 95% confidence range
For statistical tests used see section 7.2.3.

TABLE 7.29

FETAL WEIGHT, HEAD WEIGHT AND HEAD ACETYLCHOLINESTERASE ACTIVITY OF 11 DAY FETUSES

No.	Fetal wt. (mg)	Head Wt. (mg)	Head AChE (nmol/min/mg)
C1	35,5	9,2	0,881
C2	15,3	7,2	1,012
C3	28,6	9,1	0,914
C4	28,6	10,3	0,783
C5	36,1	8,4	0,946
C6	44,8	8,8	0,816
C7	23,4	7,7	1,077
C8	36,1	14,2	0,914
C9	33,0	7,7	0,979
C10	31,8	9,9	0,979
C11	29,6	7,9	0,979
C12	34,3	7,9	0,979
C13	33,0	7,6	0,914
C14	26,7	6,8	0,848
C15	23,8	7,7	0,979
Median	30,8	8,4	0,946
±	(26,7-34,6)	(7,7-9,4)	(0,881-0,979)

TABLE 7.30.

FETAL WEIGHT, HEAD WEIGHT AND HEAD AChE ACTIVITY OF 12 DAY FETUSES

No.	Fetal weight (mg)	Head weight (mg)	Head AChE (nmol/min/mg)
C1	61,9	13,9	1,109
C2	63,2	18,3	1,109
C3	78,7	31,1	1,109
C4	61,6	20,5	1,044
C5	81,5	26,3	1,109
C6	70,9	17,0	1,109
C7	60,6	18,3	1,175
C8	63,1	13,9	1,240
C9	73,0	23,0	1,109
C10	76,1	19,1	1,175
C11	60,2	13,9	1,207
C12	56,1	14,7	1,175
C13	69,7	19,1	1,175
C14	77,0	23,5	0,848
C15	65,3	12,6	1,272
C16	70,4	21,2	(spoilt)
C17	99,0	25,0	0,946
C18	66,2	21,7	1,175
C19	59,0	14,7	1,272
C20	60,1	14,8	1,109
C21	73,5	17,6	1,175
C22	64,7	17,0	1,175
Median ±	67,8 (60,3-81,5)	18,7 (16,5-21,0)	1,142 (1,012-1,224)
p	<0,00003*	<0,00003*	<0,00003*

* comparison with data in Table 7.29.

Median head AChE activity of 11 day control fetuses of 0,946nmol/min/mg was lower than for 12 day control fetuses (1,142nmol/min/mg, <0,00003.

Discussion

The decrease in fetal and head weight was again in keeping with a growth inhibitory effect of cyclophosphamide. A rapid onset of growth depression must have occurred to explain this effect. This contrasts with dosing later in gestation (day 12) where no significant growth retardation was apparent 24h after administration.

In the fact of a decrease in fetal and head weight the increase in head AChE activity appeared to add further support to the likelihood that cyclophosphamide had an effect on AChE which was independent of the growth inhibitory effect. (The increase was unlikely to be explicable on the basis of retarded development as younger fetuses had a lower head AChE activity).

A possible explanation for the increased head AChE activity was suggested by the observation that the brains of some of the cyclophosphamide treated fetuses had small areas of haemorrhage (haemorrhagic necrosis has been noted in the literature - see section 1.3). Because AChE activity in 12 day fetal heads is low it was possible that blood AChE was accounting for the higher activity (median AChE activity of 18 day fetal blood was found to be 1,71nmol/min/mg - see section 3.12). The haemoglobin concentration in heads of treated fetuses was therefore compared with that of controls as an index of the amount of blood present (see section 3.12).

7.4.9. Cyclophosphamide, 20mg/kg per mouse, administered on day 10 p.c. (Sacrifice day 12 p.c.) Comparison of head haemoglobin concentration with that of controls

Haemoglobin determination was performed using the cyanmethemoglobin method (see section 3.12). Absorbance at 540nm of head homogenate (20mg/ml) from treated fetuses was compared with controls. Results are given in Table 7.31 (p. 240).

Discussion

As noted in previous experiments the fetal weight and head weight of cyclophosphamide-treated fetuses were significantly lower than that for controls ($p<0,001$). The median absorbance at 540nm for cyclophosphamide treated fetal heads was 0,012, and for untreated fetuses 0,004, ($p<0,001$) i.e. 2,86 times higher for the treated group. This was in keeping with a significantly greater amount of blood in treated fetal heads, which was therefore the likely explanation for the increased AChE activity. (Because of the minute size of the 12 day fetuses it was impossible to collect sufficient blood for AChE determination. However, as already mentioned, median AChE activity of 18 day fetal blood was 1,71 nmol/min/mg which was higher than that of 12 day fetal brains).

7.4.10. Cyclophosphamide, 20mg/kg per mouse, administered on day 10 p.c. (Sacrifice day 14 p.c.) Comparison of head haemoglobin concentration with that of controls

This experiment was performed in an attempt to determine whether the absence of an increase in AChE in the treated group compared with controls 4 days after dosing in section 7.4.6. was related to the presence or absence of blood. Haemoglobin determination was performed using the cyanmethemoglobin method (see section 3.12). Absorbance at 540nm of head homogenate (20mg/ml) from treated fetuses was compared with controls. Results are given in Table 7.32 (p. 241).

There was no significant difference in absorbance at 540nm between tests

TABLE 7.31.

RESULTS OF 20mg/Kg CYCLOPHOSPHAMIDE ON DAY 10 p.c. (SACRIFICE DAY 12 p.c.). COMPARISON OF FETAL WEIGHT, HEAD WEIGHT AND HEAD ABSORBANCE AT 540nm

No.	Fetal Wt. (mg)	Head Wt. (mg)	Head Absorbance (540nm)
T1	35,1	11,00	0,0128
T2	33,2	8,8	0,0180
T3	41,3	14,4	0,0102
T4	45,5	12,5	0,0120
T5	42,8	11,9	0,0133
T6	36,1	9,9	0,0123
T7	44,1	8,9	0,0121
T8	44,1	14,1	0,0087
T9	42,0	11,6	0,0090
T10	50,3	12,1	0,0108
Median ±	42,00 (37,6 - 45,5)	11,6 (10,0 - 12,3)	0,0118 (0,0102 - 0,0141)
C1	66,0	17,6	0,0015
C2	60,0	14,4	0,0033
C3	64,3	19,2	0,0025
C4	61,5	19,3	0,0072
C5	62,5	16,0	0,0047
C6	57,6	14,4	0,0061
C7	55,6	14,3	0,0045
C8	69,4	21,5	0,0017
C9	70,2	18,4	0,0057
C10	59,5	16,8	0,0044
Median ±	62,5 (58,8 - 66,4)	16,85 (15,0 - 19,2)	0,0043 (0,003 - 0,006)
p	0,001	p<0,001	p<0,001

T A B L E 7.32.

RESULTS OF CYCLOPHOSPHAMIDE 20mg/kg ON DAY 10 p.c. (SACRIFICE DAY 14 p.c.) COMPARISON OF HEAD ABSORBANCE AT 540nm

	TEST	CONTROL
NO.	HEAD ABSORBANCE (540nm) 20mg/ml head homogenate	HEAD ABSORBANCE (540nm) 20mg/ml head homogenate
1	0,0058	0,0059
2	0,0073	0,0082
3	0,0087	0,0077
4	0,0055	0,0065
5	0,0052	0,0042
6	0,0058	0,0095
7	0,0081	0,0061
8	0,006	0,0063
9	0,0081	0,0051
10	0,0055	0,006
11	0,0082	0,0063
12	0,0120	0,0045
13	0,007	0,0053
MEDIAN	0,00695 (0,0058 - 0,0084)	0,0062 (0,0053 - 0,0073)
p	NS	

95% confidence limits are in brackets

and controls.

Discussion

The absence of a greater head AChE activity in the treated group 4 days after dosing (section 7.4.6.) was therefore not associated with in increase in head haemoglobin. This suggested considerable repair with resorption of blood had occurred after 4 days, which contrasted with the findings 2 days post administration of the teratogen (section 7.4.8. and 7.4.9).

7.4.11. Cyclophosphamide 15mg/kg administered on day 10 p.c. (Sacrifice day 18 p.c.) Determination of choline acetyltransferase activity

This experiment was performed to compare the effect of cyclophosphamide on ChAT activity with that on AChE, where a decrease in activity accompanied a lower fetal and brain weight (see section 7.4.2).

Results and discussion

See Tables 7.33T and 7.33C for original data (pp. 434-436).

Summary of results in Table 7.34.

There was a 26,5% resorption rate in the treated group (a higher incidence than with 12,5mg/kg) compared with 0% in controls ($p<0,02$) and a 8,8% incidence of IUD's compared with 0% in controls. One fetus treated with cyclophosphamide had exophthalmos. There were no abnormalities in the control group.

There was a significantly lower fetal weight in the treated group compared with controls (median fetal weight = 0,8214g and 1,062g respectively, $p<0,00006$). Brain weights were also significantly lower in the test group compared with controls (median = 57,0mg and 68,85mg respectively, $p<0,00006$) as was the brain ChAT activity (median 568 d.p.m. and 734 d.p.m. respectively, $p = 0,008$). A similar effect was noted with AChE (see section

TABLE 7.34.

SUMMARY OF RESULTS OF CYCLOPHOSPHAMIDE 15mg/kg ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 18 p.c.)

	TEST	CONTROL	p
Total Conceptions	34	21	-
Resorptions (%)	26,5	0	< 0,02
Intra-uterine Deaths (%)	8,8	0	< 0,1
Abnormalities (%)	4,5	0	NS
Median Fetal Weight (g)	0,8214 (0,7936-0,8579)	1,062 (1,036-1,088)	<0,00006
Median Brain Weight (mg)	57,0 (55,1-59,0)	68,85 (67,1-71,0)	<0,00006
Median ChAT Activity d.p.m.	568 (505-668)	734 (647-820)	p=0,008

Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

7.4.4.) and is consistent with the growth inhibitory effect of cyclophosphamide (there is a slow continuous rise in ChAT activity in brain tissue during development (Bus and Gibson, 1974). Developmental retardation resulting from cyclophosphamide administration would therefore be anticipated to be associated with a lower enzyme activity compared with controls).

(As was noted with previous ChAT experiments (see section 6.6.) a wide variation in ChAT activity between brain samples was apparent).

7.4.12. Cyclophosphamide 20mg/kg per mouse, administered on day 10 p.c. (Sacrifice day 12 p.c.)

This experiment was performed to compare the effects of cyclophosphamide on ChAT activity with those on AChE activity. Maternal in vivo administration of cyclophosphamide 20mg/kg on day 10 resulted in an increased head AChE activity, thought to be on the basis of increased blood (see sections 7.4.8 and 7.4.9). As blood does not contain significant quantities of ChAT this experiment was performed to assess the effect of cyclophosphamide on head ChAT activity independent of the effect of blood and to therefore further elucidate the cause for the increased AChE activity.

See Tables 7.35T and 7.35C for original data (pp. 437-439).
A summary of results is given in Table 7.36.
There were three resorptions in the test group and two IUD's. In the one dead fetus the whole brain looked haemorrhagic. In two of the viable fetuses small haemorrhagic areas were apparent macroscopically in the brains and blood stained liquor was apparent with two others. There was one resorption in the control group and no abnormalities were apparent. A significant decrease in fetal and brain weight in the treated group ($p<0,00006$), as noted in previous experiments with this dose, was appa-

TABLE 7.36.

SUMMARY OF RESULTS OF CYCLOPHOSPHAMIDE

20mg/kg ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 12 p.c.)

	TEST	CONTROL	p
Total Conceptions	25	23	-
Resorptions (%)	12	4	NS
Intra-uterine Deaths (%)	9,1	0	NS
Abnormalities (%)	20	0	<0,05
Median Fetal Weight (g)	0,052 (0,048-0,055)	0,066 (0,063-0,07)	<0,00006
Median Head Weight (mg)	19,15 (17,1-21,3)	25,45 (24,0-28,2)	<0,00006
Median ChAT Activity d.p.m.	661,8 (633 - 701)	729 (675 - 779)	NS (p=0,056)

Figures in brackets are the 95% confidence range

For statistical tests used see Section 7.2.3.

rent. Head ChAT activity of treated fetuses was 662 d.p.m. which was lower than controls (729 d.p.m.), but only at the p = 0,06 level.

Discussion

The low ChAT activity in 12 day fetal heads compared with 18-day fetal mouse brains is apparent (median blank activity was 676 d.p.m. and was not subtracted when analysing results because of the low total activity.)

The lower head ChAT activity in the treated group as opposed to the higher AChE activity noted in the previous experiments lends further support to the suggestion that the higher AChE was due to the presence of increased blood. If another effect, independent of a haemorrhagic process, had caused the increased AChE activity a similar rise in ChAT activity might have been acticipated. (If, for example, the cause of the higher AChE activity was exuberant repair of cholinergic neurons, then the synthetic enzyme of acetylcholine, ChAT, would also be expected to be increased).

Despite the absence of statistical significance the lower head ChAT activity in the treated group compared with controls would be in keeping with the growth inhibitory effect of cyclophosphamide. (The very low ChAT activity and wide variation in activity between samples reduced the likelihood of demonstrating a significant difference).

7.5. ANALYSIS OF THE ISOENZYMES OF FETAL BRAIN ACETYLCHOLINESTERASE : CYCLOPHOSPHAMIDE, 12,5mg/kg PER MOUSE ADMINISTERED ON DAY 10 p.c. (SACRIFICE DAY 17 p.c.)

Separation and densitometric analysis of the isoenzymes of AChE were performed as described in Chapter 5. The isoenzymes from 18 brain homogenates of treated fetuses were compared with those from 18 controls.

Results

Five isoenzymes were present in all test and control samples. No obvious difference was apparent on visual inspection of the gels between tests and controls, and the isoenzyme pattern was the same as that observed in 18- and 19-day fetuses in previous experiments.

Peak heights were measured from the best apparent baseline. The same three methods of analysis of the densitometric results as described for vitamin A in section 6.7 were used. Statistical analysis was performed by the Tukey modification of the Willcoxon signed ranks test and the Mann Whitney U test. In addition, some of the peaks were analysed by the Kolmogorov-Smirnov two-sample test.

A summary of the results obtained using the three methods of analysis is given in Table 7.37.

Discussion

As expected from the decreased brain AChE activity (using the Ellman technique, section 7.4.2) of animals treated with cyclophosphamide, the sum of the peak heights of the isoenzymes was apparently lower for tests than for controls, but this did not reach statistical significance. The median peak height of band 4 for treated fetuses (21mm) was significantly lower than for control fetuses (31mm, $p<0,02$), as were the median ratios using methods 2 and 3. Also of interest was that for band 5 the median peak height appeared to be slightly higher than for controls (43,25mm and 39mm respectively), although this was not significant.

7.6. SUMMARY AND DISCUSSION OF CYCLOPHOSPHAMIDE STUDY

In this study a dosage range from 7,5mg to 20mg/kg cyclophosphamide per

T A B L E 7.37.

SUMMARY OF DENSITOMETRIC RESULTS

CYCLOPHOSPHAMIDE, 12,5mg/kg, ADMINISTERED ON DAY 10 p.c.

peaks		Method 1 median peak hts. mm	Method 1 p	Method 2 median ratio	Method 2 p	Method 3 median ratio	Method 3 p
1	C	35	NS	0,525	NS	0,6975	NS
	T	33,63		0,520		0,6950	
2	C	75	NS	1,120	NS	1,500	NS
	T	73,5		1,168		1,455	
3	C	68,5	NS	-	-	1,365	NS
	T	64		-		1,280	
4	C	31	<0,02	0,4675	<0,02	0,6375	<0,02
	T	21		0,3350		0,4250	
5	C	39	NS	0,5600	NS	0,7650	NS
	T	43,25		0,6775		0,8725	
Sum of 1-5	C	250,5	NS	3,710	NS	5,030	NS
	T	235,8		3,673		4,798	

For derivation of medians and p values see section 6.7.

mouse was used, which resulted in a spectrum of observations from growth inhibition alone at lower doses to gross malformations and embryolethal effects at higher doses. Dosing on a single day was suitable for producing embryotoxic effects. The highest dose used of 20mg/kg appeared to be below the maternal M.T.D. and no toxicity was observed in the dams in any of the experiments. This lends support to a direct effect of the drug on the embryo.

As was noted with vitamin A, the incidence of resorptions increased with an increase in dose, reaching 78% with 20mg/kg administered on day 10 p.c. (see Fig. 7.3).

The embryolethal effect of cyclophosphamide must have been manifest after day 12 because there was no significant increase in mortality noted in fetuses examined on day 12, but presumably occurred shortly after this to account for the high incidence of resorptions, and not later intra-uterine deaths, when fetuses were examined on day 18 p.c. Dosing earlier in gestation with 12,5mg/kg on day 8½ p.c. was associated with a substantial increase in the incidence of resorptions of 94,7%, compared with 11,1% on day 10. (A similar dose-dependent increase in resorption rate was noted in Swiss-Webster mice when cyclophosphamide was administered intraperitoneally on day 10 p.c. by Gibson and Becker (1968), although a low dose of 5mg/kg was obviously embryolethal causing a 20% resorption rate, which increased to 37% on doubling the dose to 10mg/kg. Cyclophosphamide, 20mg/kg resulted in a 67% resorption rate, whereas the same dose given a day earlier on day 9 caused a 97% resorption rate).

Intra-uterine deaths

A dose of 7,5mg/kg was not associated with a significant incidence of IUD's but there was a small increase from 11,1% to 15% with a change

in dose from 12,5 to 15mg/kg (a higher dose of 20mg/kg exerted an embryolethal effect earlier resulting predominantly in resorption rather than later intra-uterine death). The age of dead fetuses was judged to be about 14 days, which, taking the growth inhibitory effect of cyclophosphamide into account would indicate that death probably occurred 4 to 5 days after administration.

Gross Morphological abnormalities

These were less frequently observed than with vitamin A. Furthermore, in contrast to vitamin A, the cyclophosphamide dose-response curve for gross malformations with lower doses was to the right of that for embryolethality (although it must be emphasised that these were gross, and by inference not total abnormalities). Gibson and Becker (1968) and Gebhardt (1970) also noted that smaller doses of 5 to 10mg/kg resulted in an increased incidence of resorptions with no discernible abnormalities. The results in this study (section 7.4.1. where there was a 100% abnormality and 78% resorption rate) suggest that with the higher dose of 20mg/kg, however, the dose response curve for malformations has moved to the left of the mortality curve. The predominant malformations observed with dosing on day 10 were defects of the eye (prominent eyes or exophthalmos) and craniofacial deformities. Areas of cerebral haemorrhage were evident in some 12-day fetuses after 20mg/kg on day 10, which is in keeping with the described CNS effects (Kreybig and Smidt, 1967; Wendler, 1979).

Fetal weight and brain weight

There was a dose-dependent decrease in fetal weight and brain weight (see Fig. 7.2), which was in keeping with the growth inhibitory effect of the teratogen. The reduction appeared to be independent of a teratogenic effect, with the dose response curve for growth inhibition to the left of that for embryolethality and malformations. A similar finding was

noted by Bus and Gibson (1973) with ifosfamide, a structural analogue of cyclophosphamide.

Acetylcholinesterase

In 18- and 19-day fetuses there was a decrease in AChE activity. This occurred in association with a decrease in fetal and brain weight, and with doses that were teratogenic, as judged by gross morphological abnormalities in some of the fetuses. A dose of 7,5mg/kg on day 10 which did not appear to be teratogenic, but which was associated with a decrease in fetal weight and brain weight, did not result in any change in AChE compared with controls. Although the decrease in fetal weight and brain weight was dose dependent this was not the case with AChE (see Fig. 7.2). The decrease appeared to be maximal with a dose of 12,5mg/kg on day 10 p.c., with no further decrease at a dose of 20mg/kg. The possibility of an effect on AChE, perhaps teratogenic, independent of a growth retarding effect, was raised but not proven.

Examination of fetuses 2 days after dosing on day 12 revealed an increase in head AChE despite a decrease in fetal weight and head weight. This was associated with a 2,86 times greater head haemoglobin suggesting that blood was responsible for this increase, probably as a result of a haemorrhagic process. Although examining fetuses shortly after administration of the teratogen should have reduced the influence of growth inhibitory effects and repair processes, the substantial amount of blood which resulted in an increase in AChE activity confounded the possibility of assessing possible changes in AChE independent of the above two parameters.

The absence of an increase in head AChE activity in the treated group 4 days after dosing (section 7.4.6) was not associated with an increase in haemoglobin (section 7.4.10) and suggested that considerable repair with resorption of blood had occurred by this stage. The absence of a decrease

in head AChE in this experiment despite a significant growth inhibitory effect was also interesting. A possible mechanism could have been exuberant repair of cholinergic neurons (compare vitamin A) which masked a decrease in AChE associated with the growth inhibitory effect. The absence of increased brain haemoglobin in the treated group tended to exclude the possibility that some residual blood accounted for the absence of a decrease in AChE activity.

The absence of an increase in head AChE on day 13, after administration of cyclophosphamide 20mg/kg on day 12 p.c., suggested the absence of a haemorrhagic process. This is consistent withe the principle that the fetal CNS is likely to be less susceptible to the embryotoxic effects of this dose later in gestation.

Choline acetyltransferase

As with AChE, the median brain ChAT activity of 568 d.p.m. in fetuses treated with 12,5mg/kg on day 10 was lower than that of controls (734 d.p.m., $p = 0,008$). This was also associated with a significant decrease in fetal weight and brain weight and was consistent with the growth inhibitory effect of the teratogen. Head ChAT activity in 12 day fetuses was also lower than controls (section 7.4.12) which contrasted with the higher AChE activity and was further indirect evidence that the higher AChE was due to the presence of blood.

The only significant finding on examination of the isoenzymes of AChE was a lower peak height for band 4 for treated fetuses (21mm) compared with 31mm for controls ($p<0,02$). This is clearly different to the findings of a greater peak 5 and sum of the peak heights after vitamin A administration on day 10 (section 6.7) and demonstrates different effects of the two teratogens on the isoenzymes.

7.7. CONCLUSIONS

It is concluded from this study that:

a) A dosage regimen of 20mg/kg cyclophosphamide or less was not associated with significant maternal toxicity.

b) *In vivo* maternal administration of cyclophosphamide resulted in a dose-related increase in the incidence of resorptions and intra-uterine deaths, although a high dose of 20mg/kg resulted predominantly in resorptions rather than later intra-uterine deaths.

c) Administration of cyclophosphamide on day 8½ of gestation resulted in a steep increase in resorption rate.

d) There appeared to be a dose dependent decrease in fetal weight and brain weight (Fig. 7.2), which was in keeping with the growth inhibitory effect of cyclophosphamide.

e) The dose-response curve for growth inhibition was to the left of that for malformations and embryolethality and appeared to be independent of a teratogenic effect.

f) Cyclophosphamide, independent of its embryotoxic effects, did not influence fetal brain AChE activity.

g) Cyclophosphamide administration of 12,5mg/kg or more on day 10 resulted in a decrease in brain AChE and ChAT activity in 18- or 19-day fetuses and was associated with a decrease in fetal weight and brain weight.

h) The increase in head AChE activity in fetuses examined 2 days after dosing in the face of an increased haemoglobin but decreased ChAT activity suggest that the increase was due to blood, which is compatible with a haemorrhagic process.

i) Repair processes appear to have been completed by 4 days after administration as judged by an absence of an increase in head haemoglobin or AChE compared with controls.

j) The administration of 12,5mg/kg cyclophosphamide was associated with a significantly lower peak height for isoenzyme band 4 (21mm), compared with controls (31mm, $p<0,02$).

k) Fetal brain or head AChE activity was influenced by growth inhibition and the amount of blood present and does not appear to offer any particular advantage over standard teratological parameters as a marker of teratogenicity.

CHAPTER 8

A STUDY OF THE EFFECTS OF IN VIVO MATERNAL ADMINISTRATION OF SODIUM VALPROATE ON THE C3H MOUSE FETUS

8.1. INTRODUCTION

A discussion on sodium valproate as a teratogen, with particular reference to its effects on the developing CNS, has been given in section 1.4.

The aims of this study were:

i) To investigate the effects of sodium valproate when administered during embryonic development, including an important period of CNS development, on gross fetal parameters in C3H mice, including embryolethality, gross morphological abnormalities, fetal weight and brain weight; and

ii) to compare these with the effects on brain AChE and its isoenzymes;

iii) to compare the effects with those observed using vitamin A and cyclophosphamide in the previous studies, and further evaluate AChE as a potential brain biochemical marker of teratogenic injury;

iv) to elucidate the teratogenic mechanisms of sodium valproate with particular reference to the central nervous system.

8.2 MATERIALS AND METHODS

8.2.1. Experimental mice

Timed matings were performed with young healthy virgin female C3H mice aged 8 - 12 weeks weighing 19 - 23g, as described in section 2.4, and kept under standard experimental conditions (section 2.3). Two-hour

matings were performed. Pregnant females were randomly divided into test and control groups.

8.2.2. Drug administration

Sodium valproate was dissolved in sterile water and administered by subcutaneous (s.c.) injection. The doses used were 400 - 800mg/kg/day on 2 or more days from day 8 - 12 p.c. A s.c. injection of an equal volume of distilled water was administered to controls.

8.2.3. Records of maternal and fetal parameters and statistical analysis

Maternal Parameters

The weight on the day of mating, day of dosing and the day of sacrifice and the physical state of each mouse after dosing and before sacrifice were recorded.

Fetal Parameters

Resorptions, intra-uterine deaths, gross morphological abnormalities, fetal weights, brain weights, brain AChE activity, and in some instances AChE isoenzymes were investigated.

Statistical analysis

The incidence (not percentages) of resorptions, intra-uterine deaths and morphological abnormalities was compared with controls using the Chi-square two-tailed test. Fetal weights, brain weights and brain AChE activity were compared with controls using the Mann-Whitney U two-tailed test. Medians and 95% confidence limits were calculated from the Walsh averages, after Tukey (Steinijans and Diletti, 1983).

8.3. PRELIMINARY EXPERIMENTS

The aims of these experiments were:

i) To determine the susceptibility of C3H mice to sodium valproate,

ii) to find a suitable dosage range and times of administration of the teratogen for subsequent experiments with brain AChE;

iii) to determine whether the doses used were less than the M.T.D. for the mothers, and;

iv) to determine whether sodium valproate per se affects brain AChE activity.

Guided by doses used in NMRI mice (Nau et al., 1981b), where a dose dependent increase in exencephaly and resorption rate was demonstrated with s.c. administration of sodium valproate, 4 dosage regimens were tried:

i) 400mg/kg/day s.c. on days 8 and 9 p.c.

ii) 400mg/kg/day s.c. on days 10, 11 and 12 p.c.

iii) 800mg/400/400/kg/day s.c. on days 10, 11 and 12 p.c.

iv) 520mg/kg/day s.c. on days 8 and 9 p.c.

No controls were used for this preliminary study as this was felt to be wasteful and unnecessary.

Results are presented under each experiment and details given in Tables 8.1, 8.2, 8.3 and 8.4. (Tables of original data are at the end of the thesis, pp. 440-444).

8.3.1. Sodium valproate, 400mg/kg, administered on days 8 and 9 p.c. (Sacrifice day 18 p.c.)

See Table 8.1 (pp. 440 and 441).

There were 2 resorptions and 5 intra-uterine deaths (18%) out of 28 conceptions in the 4 mice treated. Six of 21 live fetuses (28,5%) were grossly abnormal with exencephaly, 5 of whom also had exophthalmos.

8.3.2. Sodium valproate, 400mg/kg, administered on days 10, 11 and 12 p.c.

See Table 8.2 (p.442).

There were no resorptions, one IUD, and no gross abnormalities in the 20 live fetuses.

8.3.3. Sodium valproate, 800mg/kg, administered on day 10, and 400mg/kg on days 11 and 12 p.c.

See Table 8.3 (p. 443).

Of the 6 mice treated, 2 died and the remainder demonstrated transient drowsiness and ataxia after the first dose of 800mg. Subsequent doses were therefore reduced. Two of the dams were not pregnant. In one of the 2 remaining mice all 7 fetuses had resorbed, and in the other, there were 6 resorptions and only 1 live fetus which was grossly normal.

8.3.4. Sodium valproate, 520mg/kg on days 8 and 9 p.c.

See Table 8.4 (p. 444).

There were 2 resorptions (11%), 6 intra-uterine deaths (33%) and 8 of the 10 live fetuses (80%) were grossly abnormal with exencephaly and exophthalmos.

8.3.5. Study to determine whether sodium valproate, at a dose of 400mg/kg on 2 consecutive days, was below the minimal toxic dose level for adult C3H females used

This experiment was performed as described for vitamin A in section 6.3.9. Twelve non-pregnant C3H mice weighing 22 - 25g were numbered and observed for signs of toxicity after treatment with sodium valproate 400mg/kg on 2 consecutive days. They were re-weighed 9 days after dosing and the weights compared with the pre-treatment weights (see Table 8.5).

TABLE 8.5.

MOUSE WEIGHTS ON FIRST DAY OF ADMINISTRATION OF SODIUM VALPROATE (DAY O) AND 9 DAYS LATER

WEIGHT IN GRAMS

Day O	Day 9	Weight Change
23	23	O
23	23,5	+O.5
23	23,5	+O.5
23	24	+1.O
24	24,5	+O.5
25	25	O
24	23,5	-O.5
25	24,5	-O.5
22	22	O
24,5	24	-O.5
24	24,5	+O.5
24	25	+1

Results

There was no mortality and no signs of toxicity were observed.

Table 8.5 gives the mouse wights on day O and day 9 and the difference in weight. Six of the mice gained weight, in 3 the weight remained the same and with 3 there was a reduction of O,5g. These results suggest that a dose of 4OOmg/kg sodium valproate on 2 consecutive days was below the M.T.D. for adult C3H mice.

8.3.6. Experiment to determine whether sodium valproate, independent of teratogenic effects, alters brain acetylcholinesterase activity

Sodium valproate (4OOmg/kg) on 2 consecutive days, which was the dose chosen for further studies, was administered on days 16 and 17 p.c. (when susceptibility to teratogenic effects is less likely) and fetuses sacrificed on day 18 p.c., to determine whether sodium valproate, independent of

teratogenic effects, alters AChE activity.

Results are shown in Tables 8.6T and 8.6C (pp. 445 and 446).

Summary of results in Table 8.7.

There were no gross abnormalities in the treated group and no significant difference in brain AChE activity between test and controls.

Conclusion

The absence of any difference in AChE activity between the treated group and controls suggests that sodium valproate, independent of embryotoxic effects, does not affect brain AChE activity.

8.3.7. Discussion and Conclusions of Preliminary Experiments

Sodium valproate, 400mg/kg on days 8 and 9 p.c. was obviously teratogenic with an 18% incidence of intra-uterine deaths and 28,5% of the live fetuses demonstrating exencephaly (71,5% of live fetuses were macroscopically normal). This dosing regime therefore appeared to be suitable for studies on the effects of sodium valproate on fetal brain AChE activity, and was chosen for subsequent experiments.

With treatment later in gestation at the same dose of 400mg/kg on days 10, 11 and 12 p.c. there were no resorptions or morphological abnormalities and only one intra-uterine death, suggesting no embryotoxic effect. Dosing later in gestation was therefore felt to be unsuitable.

With a dose of 520mg/kg sodium valproate on days 8 and 9 p.c. the high percentage of morphological abnormalities (80%) and the 33,3% incidence of intra-uterine deaths suggested this dose was too high for a study of brain AChE activity. The maternal toxicity and 93% resorption rate with 800mg/kg on day 10, and 400mg/kg on days 11 and 12 p.c. also indicated this was an

TABLE 8.7.

SODIUM VALPROATE, 400mg/kg, ADMINISTERED ON DAYS 16 AND 17 p.c.

	TEST	CONTROL	p
Total Conceptions	21	23	-
Resorptions (%)	5	8,7	NS
Intra-uterine Deaths (%)	5	4,3	NS
Abnormalities (%)	0	0	NS
Median Fetal Weight (g)	1,004 (0,98-1,03)	1,01 (0,99-1,03)	NS
Median Brain Weight (mg)	67,9 (66,7-69,3)	67,6 (66,0-69,7)	NS
Median AChE Activity nmol/min/mg	2,94 (2,89-3,00)	2,89 (2,82-2,96)	NS

Figures in brackets are the 95% confidence range

unsuitable dosage regimen (in NMRI mice, Nau et al., (1981b) noted a 100% resorption rate at a dose of 800mg/kg).

As expected, the above results suggested a dose-related increase in embryolethality and gross morphological abnormalities. In contrast to the findings with vitamin A and cyclophosphamide the commonest gross abnormality was exencephaly (associated in most instances with exophthalmos), although this probably reflects the earlier administration of valproate on days 8 and 9. Closure of the anterior neuropore occurs in mice on days 8-9. These findings are in agreement with those of others (Nau et al., 1981b; Bruckner et al., 1983).

A dose of 400mg/kg sodium valproate on 2 consecutive days did not appear to be toxic to adult C3H mice.

Sodium valproate, independent of embryotoxic effects, did not alter fetal brain AChE activity.

8.4. RESULTS AND DISCUSSION

Two experiments were performed with sodium valproate 400mg/kg on days 8 and 9 p.c., and 2 experiments with 440mg/kg on days 8 and 9 p.c. Tables of original data are presented at the end of the thesis (see Tables 8.8T and 8.8C; 8.10T and 8.10C; 8.12T and 8.12C and 8.14T and 8.14C (pp. 447-456).

A summary of results of each experiment is given with the text (Tables 8.9, 8.11, 8.13 and 8.15.

A summary of the effects of sodium valproate on fetal outcome in all experiments is given in Table 8.16.

Overall there was a 20,7% incidence of resorptions in the sodium valproate treated group compared with 4,7% for controls ($p<0,0005$).

Although the incidence of intra-uterine deaths of 5,1% was low in these experiments, this was significantly greater than the 1,2% for controls ($p<0,005$).

TABLE 8.9.

SUMMARY OF RESULTS OF SODIUM VALPROATE, 400mg/kg ADMINISTERED ON DAYS 8 AND 9 p.c. (SACRIFICE DAY 19 p.c.) FIRST EXPERIMENT

	TEST	CONTROL	p
Total Conceptions	32	23	-
Resorptions (%)	34	9	<0,001
Intra-uterine Deaths (%)	3	0	NS
Abnormalities (%)	10	5	NS
Median Fetal Weight (g)	1,27 (1,22 - 1,30)	1,37 (1,33 - 1,39)	<0,001
Median Brain Weight (mg)	85,4 (83,2 - 86,8)	90,5 (87,6 - 93,2)	<0,001
Median AChE Activity nmol/min/mg	3,16 (3,05 - 3,26)	3,10 (2,99 - 3,15)	NS

Figures in brackets are the 95% confidence range

T A B L E 8. 11.

SUMMARY OF RESULTS OF SODIUM VALPROATE 400mg/kg, ADMINISTERED ON DAYS 8 AND 9 p.c. (SACRIFICE DAY 19 p.c.) SECOND EXPERIMENT

	TEST	CONTROL	p
Total Conceptions	30	18	-
Resorptions (%)	17	0	<0,001
Intra-uterine Deaths (%)	10	5	NS
Abnormalities (%)	18	5	0,001
Median Fetal Weight (g)	1,23 (1,16 - 1,29)	1,32 (1,29 - 1,38)	0,02
Median Brain Weight (mg)	76,8 (71,3 - 82,0)	82,5 (78,7 - 85,5)	<0,05
Median AChE Activity nmol/min/mg	3,21 (3,14 - 3,28)	3,35 (3,25 - 3,44)	<0,05

Figures in brackets are the 95% confidence range

TABLE 8.13.

SUMMARY OF RESULTS OF SODIUM VALPROATE, 440mg/kg, ADMINISTERED ON DAYS 8 AND 9 p.c. (SACRIFICE DAY 19 p.c.)- FIRST EXPERIMENT

	TEST	CONTROL	p
Total Conceptions	26	20	-
Resorptions (%)	8	0	NS
Intra-uterine Deaths (%)	8	0	NS
Abnormalities (%)	23	0	<0,001
Median Fetal Weight (g)	1,21 (1,17-1,25)	1,29 (1,25-1,33)	0,003
Median Brain Weight (mg)	76,2 (56,1-82,4)	82,9 (81,0-85,2)	0,02
Median AChE Activity nmol/min/mg	3,34 (3,25-3,46)	3,46 (3,32-3,59)	0,09

Figures in brackets are the 95% confidence range

TABLE 8.15.

SUMMARY OF RESULTS OF SODIUM VALPROATE, 440mg/kg, ADMINISTERED ON DAYS 8 AND 9 p.c. (SACRIFICE DAY 19 p.c.) SECOND EXPERIMENT

	TEST	CONTROL	p
Total Conceptions	28	23	
Resorptions (%)	21	9	<0,001
Intra-uterine Deaths (%)	0	0	NS
Abnormalities (%)	18	0	0,001
Median Fetal Weight (g)	1,21 (1,17 - 1,25)	1,31 (1,24 - 1,36)	0,009
Median Brain Weight (mg)	79,5 (53,6 - 82,4)	84,9 (83,4 - 87,0)	< 0,001
Median AChE Activity nmol/min/mg	3,34 (3,24 - 3,46)	3,37 (3,29 - 3,44)	NS

Figures in brackets are the 95% confidence range

TABLE 8.16.

SUMMARY OF EFFECTS OF SODIUM VALPROATE ON FETAL OUTCOME

DOSE (mg/kg)	400 + 440			520		800, 400, 400	
DAY OF DOSE	8, 9			8, 9		10,11,12	
	T	C	p	T	C	T	C
TOTAL CONCEPTIONS	116	84		16	0	14	0
% RESORBED	20.7	4.7	$\ll 0.0005$	12.5	–	92.8	–
% I.U.D.	5.1	1.2	< 0.005	25.0	–	0	–
% MORPHOL. ABNORMALITIES	13.7	2.4	$\ll 0.0005$	80.0	–	0	–

p = (X^2 test)

There was a 17,4% incidence of gross morphological abnormalities in the treated group, compared with 2,4% for controls ($p<0,0005$). The commonest abnormality was exencephaly which occurred in 13% of fetuses. Exophthalmos was noted in 7% and exomphalos in 5% of fetuses.

The apparent dose-related increase in embryolethality and morphological abnormalities has been referred to in section 8.3.7. and is shown graphically in Fig. 8.1. Fetal weights were slightly, although significantly, lower in all 4 experiments for the treated groups. For example, in the first experiment, median weight of sodium valproate treated fetuses was 1,27g compared with 1,37g for controls ($p<0,001$).

Brain weights were also lower for the treated groups. For example, in the first experiment median brain weight was 85,5mg compared with 90,5mg for controls ($p<0,001$).

The lower fetal and brain weights are consistent with a growth inhibitory effect of sodium valproate. These findings are similar to those with cyclophosphamide (Chapter 7) although the growth inhibitory effect of cyclophosphamide was more pronounced.

A trend for lower brain AChE activity in the treated groups was only demonstrated in 2 experiments. In the second experiment, with 400mg/kg, median AChE activity was 3,21 compared with 3,36 for controls ($p<0,05$), and in the first experiment with 440mg/kg median AChE activity was 3,34 compared with 3,46 for controls ($p=0,09$. If exencephalic brains were excluded in the latter experiment, median brain AChE for the treated group was 3,27nmol/min/mg, $p<0,05$). The effect of 400mg/kg sodium valproate in the second experiment on fetal weight, brain weight and brain AChE activity is shown graphically in Fig. 8.2.

Fig. 8.1

THE RELATIONSHIP BETWEEN THE TOTAL DOSE OF SODIUM VALPROATE ADMINISTERED AND THE INCIDENCE OF RESORPTIONS, INTRA UTERINE DEATHS AND GROSS MALFORMATIONS.

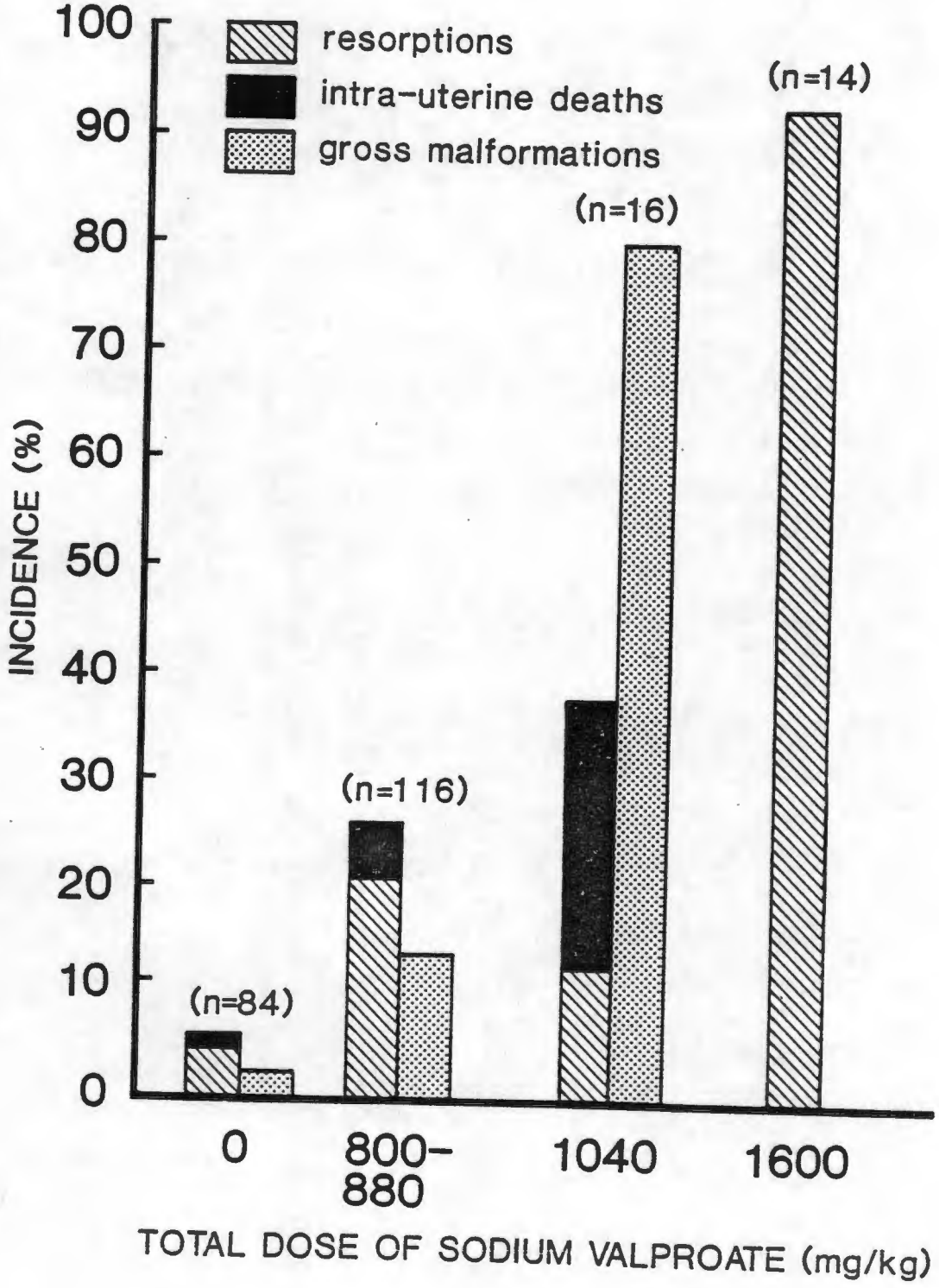
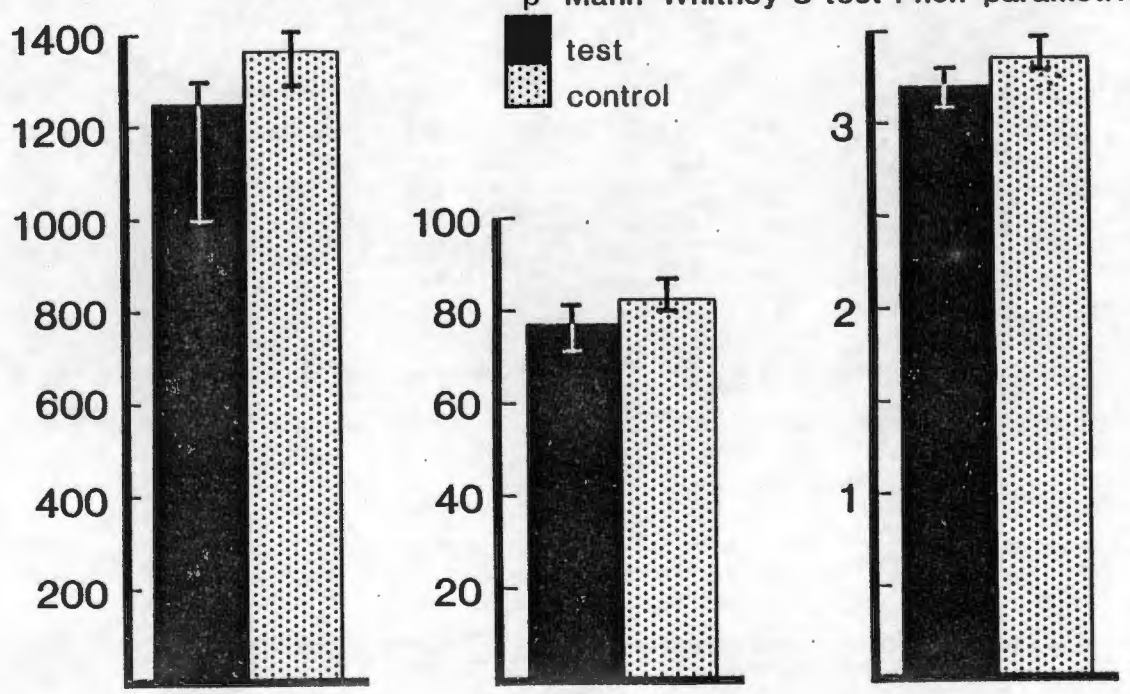


Fig. 8.2.

THE EFFECT OF SODIUM VALPROATE (400mg/Kg d.8,9) ON FETAL AND BRAIN WEIGHT AND AChE ACTIVITY

	MEDIAN FETAL WEIGHT (mg)	MEDIAN BRAIN WEIGHT (mg)	MEDIAN AChE ACTIVITY (nmol/min/mg)
p	0.05	⩽0.05	⩽0.05

p Mann-Whitney U test : non-parametric



The decrease in brain AChE activity was associated with a lower fetal and brain weight in the sodium valproate treated fetuses and could merely have been compatible with a growth retarding effect of the drug (compare cyclophosphamide, Chapter 7).

Exencephalic fetuses were also analysed separately. An analysis of fetal weight, brain weight and brain AChE of exencephalic fetuses from 2 experiments is compared with the relevant median values for Tests and Controls in Table 8.17. Although numbers were too small to demonstrate statistical significance, fetal weights and particularly brain weights of exencephalic fetuses were generally lower than those of non-exencephalic test or control fetuses. However, brain AChE activity was generally higher in exencephalic fetuses (particularly high values are evident in 4 of the 6 fetuses). In only 1 fetus (with the largest exencephalic brain) was the brain AChE activity lower than that of non-exencephalic fetuses.

The elevated AChE activity in the small necrotic looking exencephalic brains may suggest that cholinergic neurons are relatively spared in the degenerative process accompanying exencephaly, resulting in a relative preponderance of cholinergic cells. The degeneration and necrosis of brain tissue occurs secondary to the exencephaly, whatever the cause of the exencephaly, and is therefore presumably independent of the teratogenic effect of the agent used.

Although brain hemoglobin values were not determined, the higher AChE activity in exencephalic brains cannot be reasonably explained on the basis of increased blood (for example if the degenerative process was one of hemorrhagic necrosis) because the AChE activity of blood is considerably lower than that of 19-day fetal brain (see section 3.5).

In the third experiment the normal brain weight of the exencephalic fetus

TABLE 8.17.

ANALYSIS OF FETAL WEIGHT, BRAIN WEIGHT AND ACETYLCHOLINESTERASE OF EXENCEPHALIC FETUSES FROM THREE EXPERIMENTS AND COMPARISON WITH MEDIAN VALUES OF NON-EXENCEPHALIC TESTS (T) AND CONTROLS (C) IN EACH EXPERIMENT

Fetus	Fetal Wt.	Median (mg)		Brain wt.	Median (mg)		AChE	Median	
No.	mg	T	C	mg	T	C	nmol/ min/mg	T	C
1	980	1200	1310	27,8	81,6	84,7	3,59	3,33	3,29
2	1080	(n=21)	(n=21)	19,9			*		
21	1080			20,6			4,69		
22	1260			19,8			5,92		
15	1110	1200	1285	59,3	81,3	82,55	3,34	3,27	3,405
16	1050	(n=21)	(n=20)	27,0			4,73		
17	1200			23,0			*		
21	1020			22,7			*		
22	1160			39,1			6,07		
12	1190	1250	1360	74,1	77,2	82,5	2,15	3,205	3,36
13	1001	(n=20)	(n=17)	16,6			*		

* Too small for analysis
n= number of fetuses

suggests an absence of degeneration and necrosis. Acetylcholinesterase would therefore not be expected to be increased and in fact is considerably lower than that of non-exencephalic fetuses. This is in keeping with the lower AChE in fetuses exposed to sodium valproate. Further study is envisaged to confirm these findings in exencephalic brains and to elucidate the mechanisms involved.

With the timing of administration and doses of sodium valproate used an obvious teratogenic effect was demonstrated. It was therefore felt that further experimentation with different doses and times of administation was not warranted. In the absence of promising AChE changes in whole brains I did not endeavour to examine AChE changes in specific brain areas.

8.5. ANALYSIS OF THE ISOENZYMES OF FETAL BRAIN ACETYLCHOLINESTERASE : SODIUM VALPROATE, 400mg/kg, ADMINISTERED ON DAYS 8 AND 9 p.c.

Separation and densitometric analysis of the isoenzymes of AChE were performed as described in Chapter 5. The isoenzymes from 18 brain homogenates of treated fetuses were compared with those from 20 controls.

Results and discussion

Five isoenzymes of AChE were present in all Test and Control samples.
No obvious difference was apparent on visual inspection between the treated and untreated groups.
Peak heights were measured from the best apparent baseline. The same 3 methods of analysis of the densitometric results as described for vitamin A in section 6.7 were used. Statistical analysis was by the Tukey modification of the Willcoxon signed ranks test and the Mann-Whitney U test. In addition some of the peaks were analysed by the Kolmogorov-Smirnov two-sample test. A summary of the results obtained using the 3 methods is given in Table 8.18 and shown graphically in Fig. 8.3.

TABLE 8.18.

SUMMARY OF DENSITOMETRIC RESULTS

SODIUM VALPROATE, 400mg/kg, ADMINISTERED ON DAYS 8 AND 9 p.c.

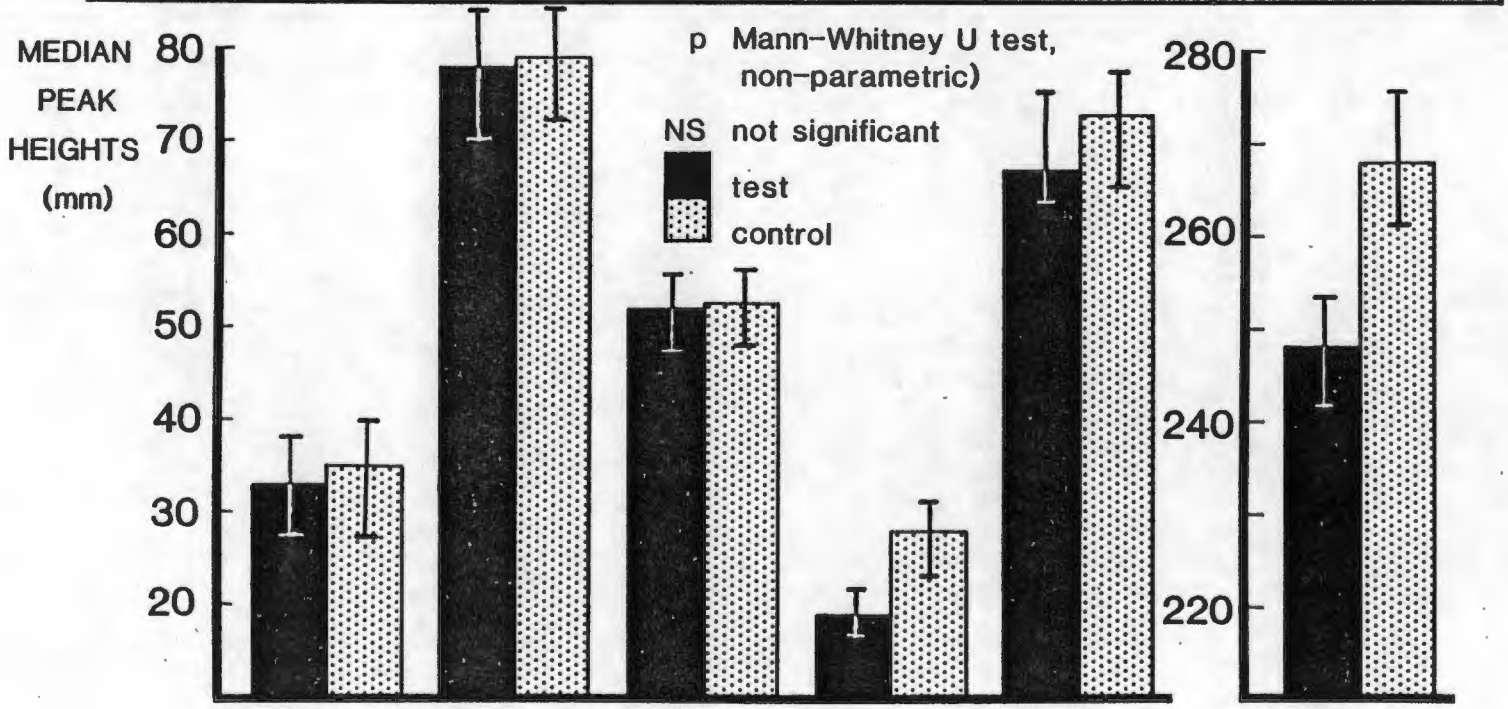
		Method 1		Method 2		Method 3	
Peaks		Median peak hts mm	p	Median ratio	p	Median ratio	p
1	T	32,88 (n=20)	NS	0,62	NS	0,615	NS
	C	35,0 (n=20)		0,67		0,66	
2	T	77,88	NS	1,465	NS	1,43	NS
	C	79,25		1,51		1,47	
3	T	52,38	NS	-	-	0,96	NS
	C	52,5		-		0,99	
4	T	19,0	<0,002	0,34	<0,02	0,34	<0,002
	C	27,75		0,54		0,52	
5	T	67,38	NS	1,265	NS	1,24	NS
	C	72,63		1,48		1,36	
Sum of 1-5	T	247,6	<0,02	3,71	NS	4,55	<0,02
	C	268,0		4,21		5,00	

For determination of medians and p values see section 6.7.

Fig. 8.3.

ANALYSIS OF SODIUM VALPROATE DENSITOMETER RESULTS

PEAK	1	2	3	4	5	SUM PEAKS 1-5
p	NS	NS	NS	<0.002	NS	<0.02



Although the medians for the test peaks were apparently lower than for controls, this was only statistically significant for peak 4 (19mm compared with 27,75mm, $p<0,002$) and the sum of peaks 1 - 5 (247,6mm compared with 268mm, $p<0,02$). Using method 1 the level of significance for peak 4 was $p<0,002$, and for the sum of the peaks, $p<0,02$. Using peak 3 as internal standard (method 2) did not improve the level of significance. Division of all the data by the mean control value for each gel (method 3) gave similar results to method 1.

The significantly lower sum of peak heights 1 - 5 for tests is consistent with the lower brain AChE activity for tests compared with controls noted in 2 of the experiments (section 8.4) using the Ellman technique.

A lower peak height for isoenzyme band 4 was also found after administration of cyclophosphamide (section 7.5). Because both drugs have a growth inhibitory effect on the fetus it is conceivable that this may be the reason for the common finding. Alternatively, the particular susceptibility of isoenzyme 4 may be the expression of some other embryotoxic effect. Whether this is the result of some similar underlying teratogenic mechanism warrants further investigation.

8.6. CONCLUSIONS

It is concluded from this study that:

a) A dosage regimen of 520mg/kg sodium valproate or less on 2 consecutive days was not associated with any apparent maternal toxicity, and 400mg/kg on 2 consecutive days was below the M.T.D.

b) There was an apparent dose-related increase in the incidence of resorptions and intra-uterine deaths.

c) There was an apparent dose-related increase in the incidence of gross morphological abnormalities.

d) The commonest gross abnormality was exencephaly.

e) At doses of sodium valproate from 400mg/kg - 520mg/kg there was a decrease in fetal weight and brain weight, which was in keeping with a growth inhibitory effect.

f) Sodium valproate, independent of its embryotoxic effects, did not influence fetal brain AChE activity.

g) Despite a decrease in fetal weight and brain weight sodium valproate 400 - 440mg/kg on days 8 and 9 p.c. was only associated with a decrease in AChE in 2 of 4 experiments.

h) Exencephalic fetuses appeared to have a higher AChE activity than non-exencephalic fetuses.

i) The administration of 400mg/kg sodium valproate on days 8 and 9 p.c. was associated with a significantly lower peak height for band 4, and the sum of the peaks 1 - 5.

j) Fetal brain AChE activity does not appear to offer any particular advantage over standard teratological parameters as a marker of teratogenicity induced by sodium valproate.

CHAPTER 9

A STUDY OF THE EFFECTS OF IN VIVO MATERNAL ADMINISTRATION OF LARGE DOSES OF VITAMIN A DURING THE PREIMPLANTATION PERIOD

9.1. INTRODUCTION

In contrast to the susceptibility to teratogenesis of embryos after implantation during the phase of organogenesis, preimplantation embryos are remarkably resistant (Tuchmann du Plessis, 1975 : 40; Austin, 1973). There are very few studies in the literature on the administration of vitamin A during the preimplantation period. Adams et al., (1961) found no evidence of any deleterious effect of excess vitamin A on the blastocyst or on subsequent development in the rabbit when administered during the preimplantation period. However, Morriss (1972) found malformations of the central nervous system in 8 out of 144 young rats after maternal administration of large doses of vitamin A before the time of implantation. The abnormalities were postulated to be due to changes in the maternal organism.

The aim of this study was firstly to determine the effects of large doses of vitamin A on C3H mouse embryos when administered during the preimplantation period. Secondly, to compare the findings with those resulting from administration of vitamin A later in gestation, and thirdly, to help elucidate the teratogenic mechanisms of vitamin A.

The first objective of the study was to determine the susceptibility of C3H mice to different doses of vitamin A administered during the preimplantation period by examining fetuses for gross morphological changes towards the end of gestation. The second objective was to determine whether embryotoxic effects were apparent in the preimplantation embryo

by (i) determining viability using the fluorescein diacetate test, which is dependent on esterase enzyme activity, and (ii) noting cell number, mitotic index and chromosome structure.

The principle of the fuorescein diacetate (FDA) test is that normal embryos are impermeable to fluorescein but permeable to the non-fluorescent derivative, FDA. The acetate groups of FDA are cleaved by cellular esterases releasing free fluorescein intracellularly and cells will maintain fluorescence when the plasma membranes are intact. Jackowski (1977) used a fluorescent dye technique to assay the viability of mouse ova, demonstrating a high correlation (0,96) between the ability of embryos to retain fluorescein and their ability to develop in culture. Damaged or dying cells quickly lose fluorescence and dead cells show none at all (Whittingham , 1981). Mohr and Trounson (1980) and Kola and Folb (1986) have used the test as an assay of blastocyst viability.

Vitamin A has been shown to interfere with mitosis in epithelial cells in vitro (Aydelotte, 1963 a, b) and in neuroepithelial cells of fetal mouse cerebral cortex after maternal treatment (Langman and Welch, 1967). Also, vitamin A and other retinoids have been shown to induce sister-chromatid exchange in fibroblast cultures (Tetzner et al., 1980). In this study the effect of vitamin A on cell number, mitotic index and chromosome structure of C3H mouse blastocysts was investigated, with a view further to elucidate the molecular mechanisms whereby vitamin A perturbs the pre- and post-implantation development of embryos.

9.2. MATERIALS AND METHODS

9.2.1. Experimental mice

The housing of mice and method of mating have been described in Chapter 2.

9.2.2. Drug administration

Vitamin A was administered by gastric intubation to the experimental animals on the third day of gestation, 60h after copulation. The following doses of the drug were used: 0 (controls), 5 000, 10 000, 15 000, 30 000 I.U. per mouse.

9.2.3. Assessment of embryotoxicity at 18 days post conception

The incidence of resorptions, intra-uterine deaths, gross morphological abnormalities and fetal weights was noted.

9.2.4. Retrieval of preimplantation embryos and assessment of viability

Twenty-one hours after treatment, i.e. 81h after copulation the mice were sacrificed by cervical dislocation. The uterine horns were exposed and the blastocysts flushed from the uteri using a modified Dulbecco's phosphate-buffered saline (PBS: Whittingham, 1971). Embryos were examined under a dissecting microscope and the number of blastocysts and morulae recorded. The fluorescein diacetate test used was that used by Kola and Folb (1986), based on the technique of Mohr and Trounson (1980).

Embryos were incubated for 1min at room temperature in a solution of FDA (Sigma, USA) in PBS (final concentration 2,5µg FDA/ml PBS). The embryos were then washed in plain PBS solution for 3-5min. Each embryo was examined at a magnification of x240 under a fluorescence microscope (Wild and Leitz, Germany), using a combination of filters which contained an excitor filter PB 450-490, chromatic beam splitter FT 510 and a barrier filter LP 520. Fluorescence was measured using a centre field light sample which was fed into a photomultiplier tube. The camera mechanism was triggered when a predetermined amount of light was received, and the time taken to this point was determined using a digital timer. Fluorescence that was just visible was sufficient to trigger the camera mechanism. A normal viable embryo took 2,8 to 3,1sec to trigger the

mechanism, whereas an embryo was regarded as non-viable if it took 13 sec or longer (Kola and Folb, 1986).

9.2.5. Cell number, mitotic index and chromosome structure

The technique used was based on that of Tarkowski (1966) and Kola (1985). Mice were injected intra-peritoneally with 2mg/kg colchicine (Lennon, Port Elizabeth, South Africa) 2h prior to sacrifice by cervical dislocation. Uterine horns were exposed and blastocysts flushed from the uteri with PBI. Viewed under a dissecting microscope the blastocysts were transferred with a Gibson P20 micropipette to a hypotonic solution of 1% trisodium citrate at room temperature and allowed to incubate for 10 - 20 min. The embryos were then transferred onto clean glass slides in a minimum amount of hypotonic solution and the preparation fixed by the drop-wise addition of a mixture of 3:1 methanol : glacial acetic acid. The optimal number of fixative drops in the case of blastocysts is 5 - 6. The air-dried preparations were then stained with 10% Giemsa (Merck, Darmstadt, Germany) solution for 7 min. Preparations were then examined by phase microscopy. The blastocyst cell number was determined by counting the nuclei. The mitotic index (number of cells in mitosis/total number of cells) was determined and metaphases were scored for chromosomal aberrations such as breaks, gaps, and translocations or rearrangements.

9.2.6. Statistical analysis

The number of resorptions, intra-uterine deaths and malformations in the treated and control groups was compared using the Chi-squared test. Fetal weights were compared using the Mann-Whitney U two-tailed test.

The Mann-Whitney U two-tailed test was used to compare the time taken to trigger the camera mechanism by viable treated and control embryos.

Cell numbers in the treated group were compared with controls using the Mann-Whitney U two-tailed test. The number of cells in mitosis in treated and control groups were compared using the chi-squared test.

9.3. RESULTS

9.3.1. The effects of vitamin A administered 60h after copulation on fetuses at 18 days p.c.

Tables of original data are presented at the end of the thesis (see Tables 9.2. - 9.7, pp. 457-468). Summary of results in Table 9.1.

Resorptions/Intrauterine deaths

The effects of increasing doses of vitamin A on embryo resorption/IUD's is shown in Table 9.1. There was no significant embryolethal effect with a dose of 15 000 IU per mouse or less. However, with 30 000 IU there was a 65% incidence of resorptions (38 out of 59 conceptions, $p<0,001$).

Malformations

Vitamin A administered 60h post copulation at a dose of 10 000 IU or greater was associated with a significantly higher incidence of gross abnormalities compared with controls (see Table 9.1). Malformations included exophthalmos, anophthalmia, microphthalmia, exencephaly, exomphalos and limb defects (see Tables 9.2 - 9.5, pp. 457-465). The highest incidence of malformations, 59%, was observed after treatment with 15 000 IU vitamin A (in 16 of 27 live fetuses, $p<0,001$).

Fetal Weight

There was no difference in fetal weight compared with controls after treatment with 5 000 and 10 000 IU vitamin A (see Table 9.1). The median fetal weight of fetuses treated with 15 000 and 30 000 IU vitamin

TABLE 9.1.

THE EFFECT OF VITAMIN A ADMINISTERED 60h POST COPULATION ON FETAL WEIGHT, EMBRYOLETHALITY AND GROSS MALFORMATIONS OF 18-DAY FETUSES

		DOSE OF VITAMIN A IN IU ADMINISTERED PER MOUSE			
	CONTROLS	5 000	10 000	15 000	30 000
No. of live fetuses	47	42	38	27	18
Median fetal wt (g)	1,057 (1,005-1,104)	1,069 (0,985-1,15)	1,042 (0,96-1,10)	0,94 (0,87-0,997)	0,825 (0,79-0,87)
p	-	NS	NS	<0,0003	<0,0003
Resorptions	3	2	1	5	38
p	-	NS	NS	NS	<0,001
IUD's	1	4	3	3	3
p	-	NS	NS	NS	NS
Malformations	1	0	6	16	5
p	-	NS	<0,05	<0,001	< 0,01

For derivation of p value see section 9.2.6.

A was 0,94g and 0,825g, respectively, compared with 1,057g for controls ($p<0,0003$ in each case).

9.3.2. The effect of vitamin A on viability of 81h blastocysts

Viability was assessed in 53 control and 68 treated blastocysts. Vitamin A, at a dose of 30 000 IU, which in the previous experiment was shown to be obviously embryotoxic, was not associated with any alteration in embryo viability (see Fig. 9.1). The median time taken to trigger the camera was 2,9sec for untreated and 2,8sec for treated embryos. There were 3 non-viable blastocysts in each group.

9.3.3. The effects of vitamin A on cell number, mitotic index and chromosome structure

Vitamin A, 30 000 IU per mouse, did not significantly alter cell number, mitotic index or chromosome structure (see Table 9.8 and Figs. 9.2, 9.3 and 9.4).

TABLE 9.8.

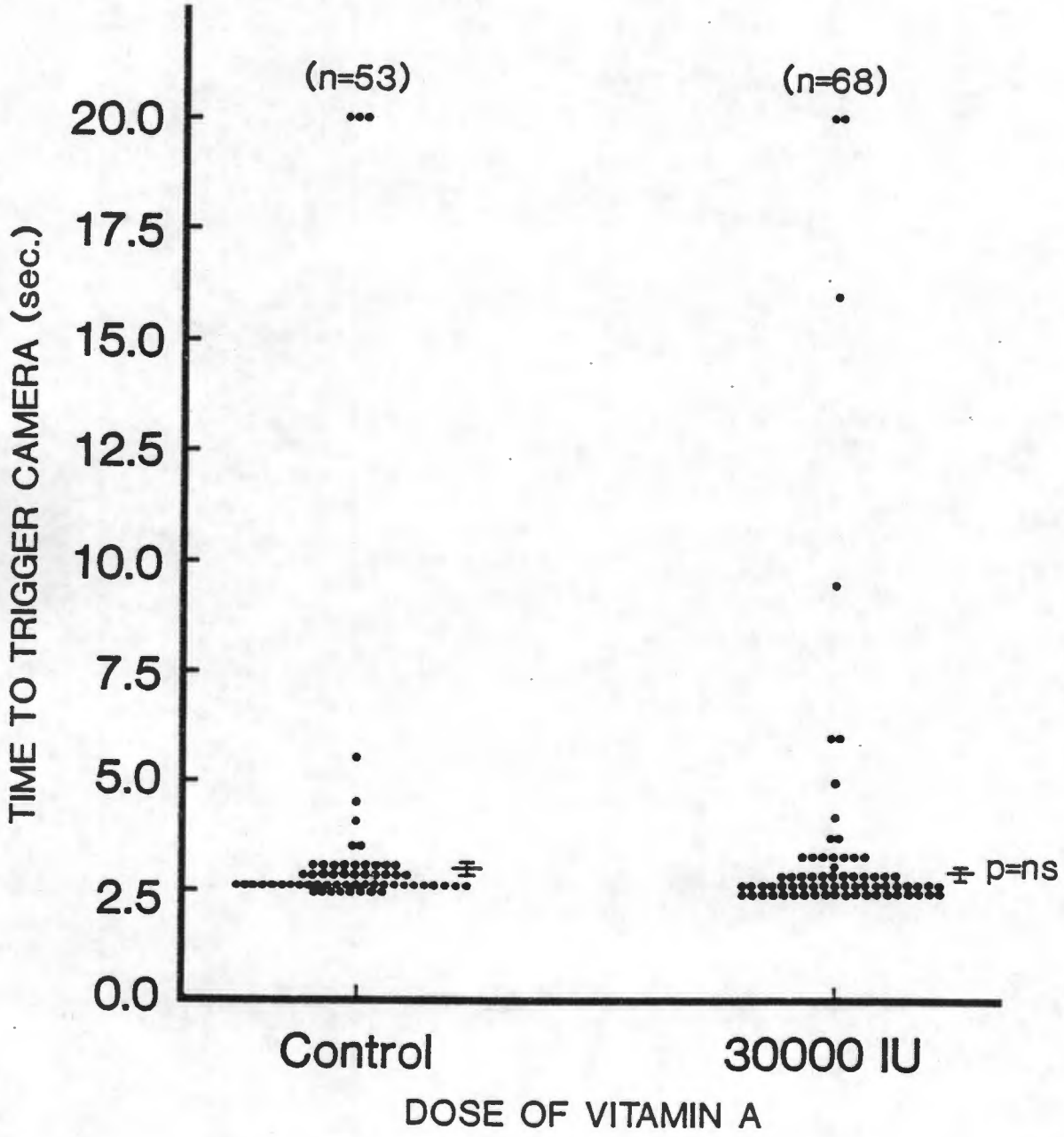
THE EFFECTS OF 30 000 IU VITAMIN A ADMINISTERED TO C3H MICE 60h AFTER COPULATION ON THE CELL NUMBER, MITOTIC INDEX AND CHROMOSOME STRUCTURE OF 81h BLASTOCYSTS

	No. of blastocysts	Cell number per blastocyst (Median)	Mitotic index	Chromosome abnormalities
CONTROL	19	34 (29-41)	6 (4-9,5)	0
TEST	34	30 * (27-34)	5 * (4-6,5)	1 break *

* N.S. Figures in brackets are the 95% confidence range.

Fig. 9.1.

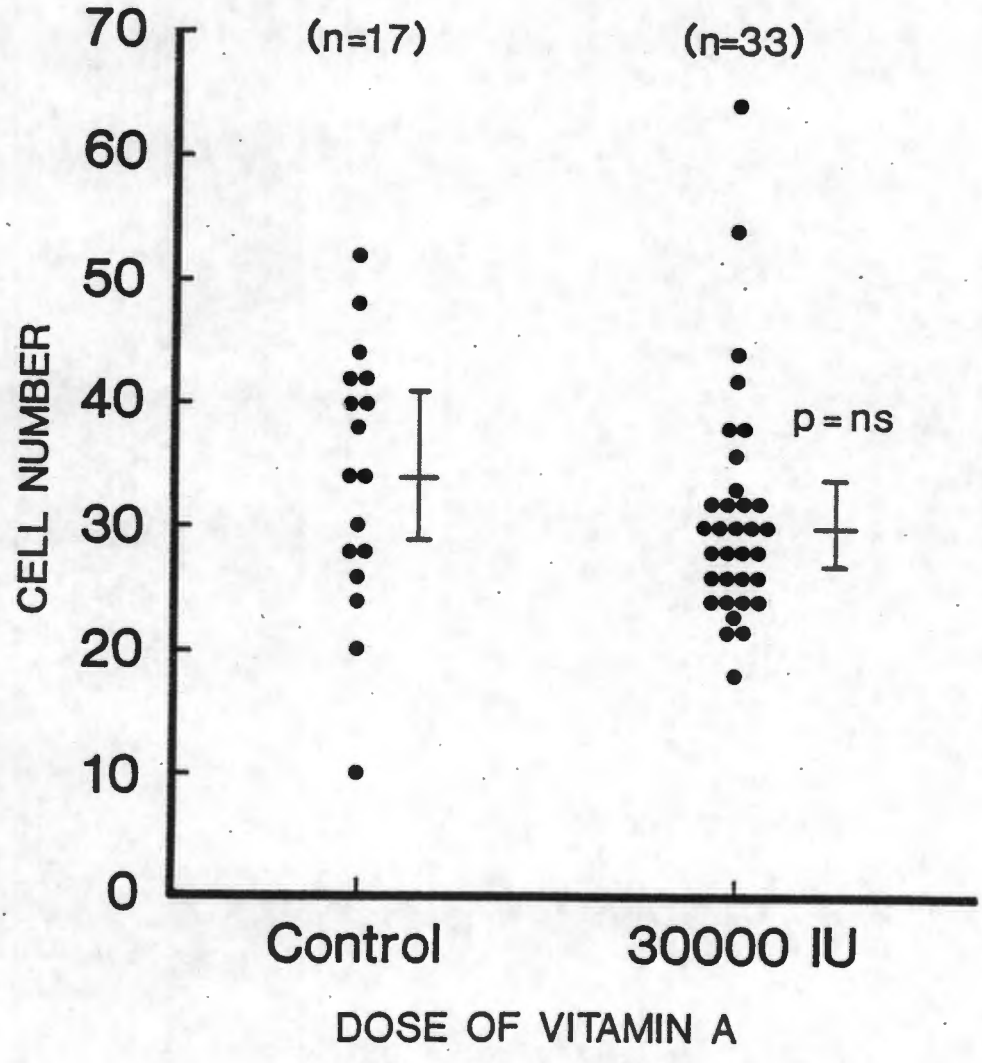
THE EFFECT OF VITAMIN A ADMINISTERED 60h POST COPULATION ON VIABILITY/ESTERASE ENZYME ACTIVITY OF 81h BLASTOCYSTS



p value determined by Mann-Whitney U Test. Error bars are 95% confidence limits. Time taken to trigger the camera mechanism is inversely proportional to the intensity of embryo fluorescence and esterase enzyme activity.

Fig. 9.2.

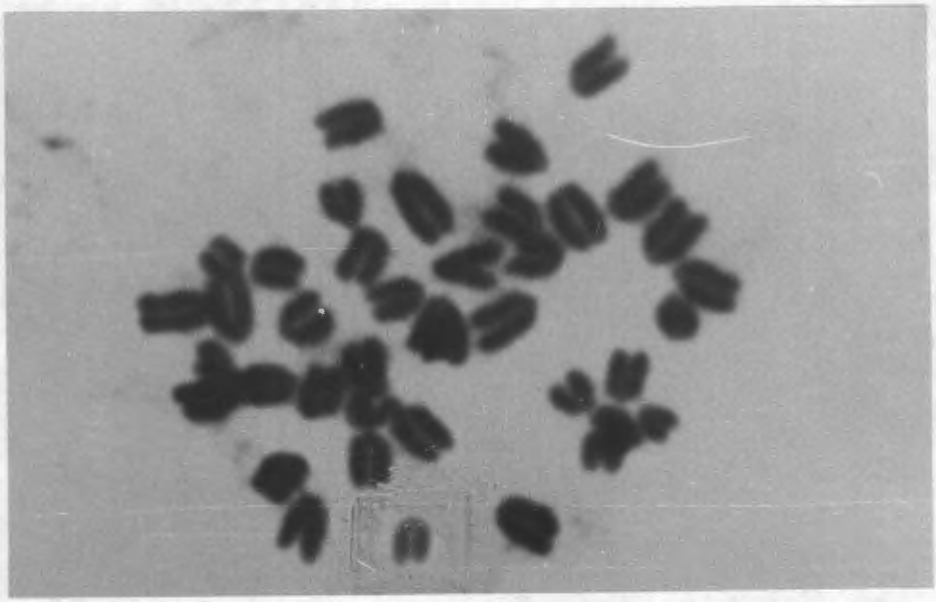
THE EFFECT OF 30 000 IU VITAMIN A ADMINISTERED TO C3H MICE 60h AFTER COPULATION ON THE CELL NUMBER OF 81h BLASTOCYSTS



p value determined by Mann-Whitney U test.

Error bars are 95% confidence limits.

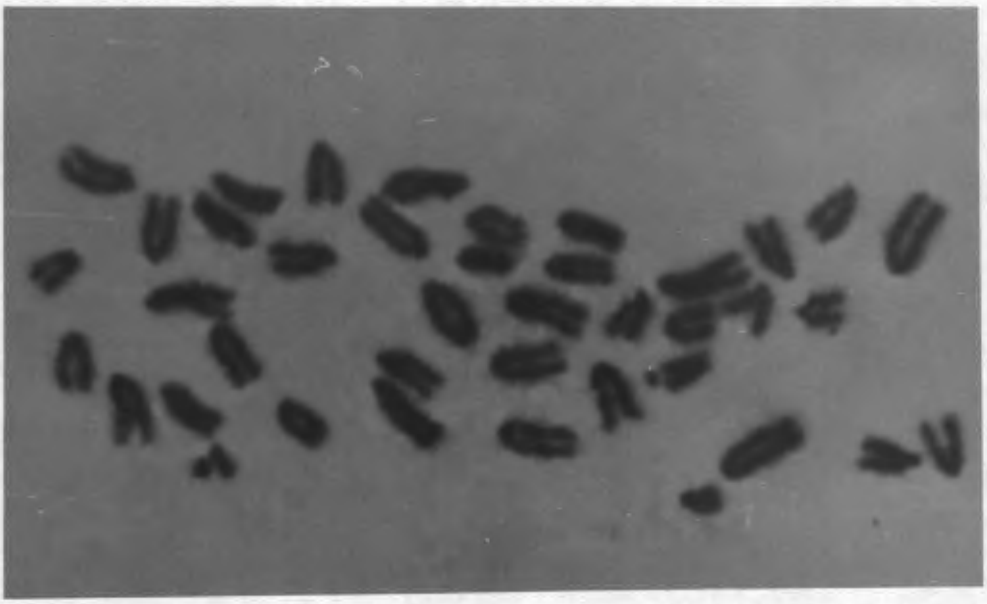
Fig. 9.3



Magnification x 1 000

Photomicrograph of a set of chromosomes from an untreated 81h blastocyst. No aberrant chromosomes are seen.

Fig. 9.4



Magnification x 1 000

Photomicrograph of a set of chromosomes from an 81h blastocyst after maternal treatment with 30 000 IU vitamin A.

No aberrant chromosomes are seen.

9.4. DISCUSSION

The findings that administration of vitamin A during the preimplantation or pre-differentiation period is associated with an obvious embryolethal effect with a dose of 30 000 IU, a decrease in fetal weight with doses of 15 000 and 30 000 IU vitamin A and gross morphological abnormalities after treatment with 10 000, 15 000 and 30 000 IU vitamin A are very interesting and to my knowledge have not been previously recorded in the mouse. The observations contrast with findings after excess vitamin A administration during the preimplantation period in the rabbit where no embryotoxic effect was demonstrated (Adams et al., 1961), and appear to be more pronounced than in the rat, where only 8 of 144 offspring were noted to have abnormalities (Morriss, 1972). The high incidence of embryotoxic effects in this study is striking. Although the reasons for this have not been investigated, a possible explanation may be on the basis of different kinetics of the vitamin in the mouse. Further study is needed to elucidate this.

Perhaps more intriguing is the apparent absence of any embryotoxic effect in blastocysts examined 22h after administration of the vitamin A according to the criteria applied in this study.

The lack of any demonstrable effect of vitamin A on blastocyst viability, cell number, mitotic index and chromosome structure is in keeping with the remarkable resistance of the preimplantation embryo to teratogenic insults and suggest that the morphological abnormalities noted in 18-day fetuses were not due to embryotoxic effects during the predifferentiation period. Although vitamin A has been shown to decrease cell proliferation (Kochar, 1968) and to inhibit mitosis in epithelial and neuroepithelial cells (Aydelotte, 1963a, b; Langman and Welch, 1967) there was no de-

monstrable evidence of this in these experiments. It is apparent the developmental aberrations were due to effects operating after the pre-implantation period. Vitamin A is known to have a long elimination half life ($t_{\frac{1}{2}}$) in the human (Rosa et al., 1984). The malformations observed, such as eye defects, limb defects, exencephaly and exomphalos were characteristic of teratogenic insults operating during the period of organogenesis (see section 1.2 and Chapter 6). This study suggests that timing was not as important in determining susceptibility as the $t_{\frac{1}{2}}$ of the teratogen, and highlights the danger of simplistically assuming resistance to teratogenesis during the predifferentiation period as the only criterion. With the retinoic acid derivitive, Etretinate, because of its long $t_{\frac{1}{2}}$ patients are advised not to fall pregnant for ½ to 2 years after discontinuing treatment (Grote et al., 1985). Similarly, Rosa et al., 1984 warn against excessive exposure to vitamin A for several months before pregnancy occurs because vitamin A is extensively stored and has a prolonged $t_{\frac{1}{2}}$. Despite this knowledge, as far as I can ascertain, this is the first animal study demonstrating the importance of this warning. The findings should also be extended to caution against the use of any potentially teratogenic drug with a long $t_{\frac{1}{2}}$.

Although the induction of chromosomal aberrations is considered an important mechanism whereby teratogens induce abnormal development (section 1.11), no serious consideration has been given in the literature to the possibility that the mechanisms by which vitamin A produces teratogenic effects may involve chromosomal defects (see section 1.2). The absence of any effects on chromosome structure in these experiments suggests that the production of chromosomal abnormalities is not an underlying mechanism in the mouse.

The absence of demonstrable embryotoxicity in the preimplantation embryos

as opposed to the postimplantation fetuses may be a further pointer to the teratogenic mechanism of vitamin A. If the mechanism was secondary to a toxic effect on cell membranes or DNA synthesis, this would have been expected to also be apparent in the preimplantation blastocysts. Although it can be argued that the resistance of the early embryo is due to the fact that the cells of the early embryo are totipotent and can "replace" damaged cells, the observation that vitamin A continues to exert its embryotoxic effects for many days may be anticipated to preclude adequate repair.

9.5. CONCLUSIONS

It is concluded from this study that:

(i) Vitamin A, 30 000 IU, administered 60h after copulation had no demonstrable effect on the viability, cell number, mitotic index or chromosome structure of 81h embryos.

(ii) Vitamin A, 30 000 IU, administered 60h post copulation was obviously embryolethal.

(iii) Vitamin A, 15 000 and 30 000 IU administered 60h post copulation was associated with a lower fetal weight compared with controls ($p<0,0003$), which is in keeping with the growth-inhibitory effect of the teratogen.

(iv) Vitamin A administered 60h post copulation at a dose of 10 000, 15 000 or 30 000 IU produced characteristic gross malformations.

(v) The above embryotoxic effects are explicable on the basis of the long $t_{\frac{1}{2}}$ of elimination of vitamin A.

CHAPTER 10

A STUDY OF THE EFFECTS OF IN VIVO ADMINISTRATION OF LARGE DOSES OF VITAMIN A ON C3H FETAL MOUSE BRAIN PROTEIN PATTERNS USING HIGH RESOLUTION TWO-DIMENSIONAL ELECTROPHORESIS

10.1. INTRODUCTION

The detection of many proteins requires methods that are independent of functional activity. Traditional methods fall far short of revealing the full complexity that is present. Two-dimensional (2D) gel electrophoresis, owing to its resolution and sensitivity, is the emerging technique that holds the greatest promise for the analysis and detection of proteins from complex biological sources (Garrels, 1980; O'Farrell, 1975). Two recent full supplements to Clinical Chemistry (1982; 1984) have been devoted to the technique. The method is based on two steps of electrophoresis of proteins in polyacrylamide gels. Isoelectric focusing is used in the first dimension to separate the proteins by charge, and sodium dodecyl sulphate (SDS) electrophoresis is used in the second dimension to separate the proteins by molecular weight. Since there is poor correlation between the charge of a protein and its molecular weight it is possible to obtain an almost uniform distribution of protein spots across the gel and the number of proteins that can be detected is in theory equal to the product of the numbers of proteins resolved by each method used above. In practice, distributions of up to several thousand well resolved proteins can be obtained from cell homogenates (Garrels, 1980). The technique is sufficiently sensitive to detect very small quantities of protein and the reproducibility permits each spot on one separation to be matched with a spot on a different separation. In most comparisons of two samples, visual inspection alone allows detection of the alteration or disappearance of a single protein in a field of up to 2 000 spots. This accounts for the

widespread usefulness of the technique without computerised data analysis (Anderson, et al., 1984).

There are a number of reasons why this technique was considered to have potential as a method for investigating the teratogenic effects and mechanisms of drugs. Firstly, the system can resolve proteins differing in a single charge and consequently can be used in the analysis of in vivo modifications resulting in a change in charge (O'Farrell, 1975). (Indeed, the system has been used for detecting point mutations in mammals (Klose, 1975; 1979) and in man (Comings, 1979). Secondly, proteins represent a class of molecules which should be particularly suitable for studying teratogenic effects in mammals at the level of macromolecules, and the proteins affected may indicate a specific teratogenic mechanism (Klose et al., 1977). There is some evidence that alkylation of proteins may constitute one of the primary targets of cyclophosphamide teratogenicity (Gurtoo et al., 1978). Thirdly, considerable qualitative differences in the composition of protein patterns occurs at different developmental stages in the embryo (Klose and von Wallenberg-Pachaly, 1976), whilst both qualitative and quantitative differences in protein spots have been shown between different strains of mice (Klose, 1979). It is therefore reasonable to expect that a teratogenic insult resulting in changes during development might produce changes in protein patterns. Finally, 2D analysis has been successfully used to detect heat-shock-induced proteins in human cells (Anderson et al., 1982), and in rat embryo fibroblasts (Welch, 1985). In a recent embryonic stress hypothesis of teratogenesis it was proposed that the induction of a heat-shock response provides a common pathway by which diverse teratogenic agents result in developmental abnormalities (German, 1984).

From the foregoing it is apparent that protein-mapping with 2D electro-

phoresis may enable detection of alterations in protein patterns when embryos bearing a teratogenically induced defect are investigated. Changes in abundance, modifications or alterations in the rate of synthesis of proteins never before observed can potentially be detected. The following preliminary study was performed with a view to future research.

10.2. MATERIALS AND METHODS

10.2.1. Experimental mice

The housing and mating of mice have been described in Chapter 2.

10.2.2. Drug administration and sacrifice

Maternal administration of 10 000 IU or 12 500 IU vitamin A was by oral gavage on day 9 p.c. As shown in Chapter 6 these doses and the time of administration were associated with obvious teratogenic effects in this mouse model.

Sacrifice was by cervical dislocation on day 11 or day 18 p.c.

10.2.3. High resolution two-dimensional electrophoresis of proteins

The technique used was based on that of O'Farrell (1975).

Sample preparation

This was adapted from Wilson et al., (1977). (The following technique was used so that autoradiography could also be performed).
10mg brain was sonicated in 22µl homogenising fluid (8M urea, 0,5% SDS, 8,71mg phenylmethylsulphonyl fluoride, 5% mercaptoethanol) and 88µl of buffer added (15mM Na_2HPO_4, 15mM NaH_2PO_4, pH7,0).

To 75µl of homogenate were added 15µl of 0,6% sodium cyanoborohydride ($NaBH_3CN$) in sodium phosphate buffer and 7,5µl of a formaldehyde solution (0,37ml formaldehyde made up to 50ml with sodium phosphate buffer). The

mixture was shaken for at least 4h and then made up to 750µl with sodium phosphate buffer. This was dialysed against deionised water for 24h with a minimum of 4 changes of water. The sample was quickly frozen in dry ice and acetone, and then resolubilised in 60µl of lysis buffer. To this was added 7,5µl of 10% Triton X-100 and ampholines (40µl pH5-7, 10µl pH3,5-10,0).

Isoelectric focusing of gels (first dimension)

The proteins were separated in the first dimension by isoelectric focusing on polyacrylamide rod gels over an approximate pH range of 4,0-7,0. Gels were made in glass tubing, sealed at the bottom with Parafilm. To a 125ml side arm flask were added 5,5g urea, 1,33ml 30% acrylamide solution, 2ml 10% Triton X-100, 1,97ml water and 0,4ml ampholines (0,4ml, pH 5-7; 0,1ml pH 3-10). The contents were mixed until the urea had completely dissolved. Ten microlitres of 10% ammonium persulphate were added and the solution degassed for 1 to 2 min. Seven microlitres of Temed were added and the mixture immediately poured into gel tubes, care being taken not to trap any air bubbles. This was carefully overlayed with a solution of 8M urea and allowed to polymerise for 1½h. The overlay solution was then poured off and replaced with 20µl of lysis buffer (9,5 M urea; 2% (w/v) Triton X-100; 2% (w/v) ampholines (1,6%. pH 5-7; 0,4%, pH 3-10); 5% w/v β - mercaptoethanol). This was overlayed with a small amount of water. The gels were allowed to set for a further 1½h. The lysis buffer, water and Parafilm were removed, 20µlof fresh lysis buffer added and overlayed with 0,02 M NaOH. The lower reservoir of a Hoefer GT3 gel electrophoresis unit for rod gels was filled with 0,01M H_3PO_4 and the upper reservoir with 0,02M NaOH. The gels were then pre-electrophoresed at 200 volts for 15 min, 300 volts for 30 min and 400 volts for 30 min. The power was turned off, the upper reservoir emptied, lysis buffer and NaOH removed from the surface of the gels, and the samples loaded and overlayed with 10µl of sample overlay solution (9 M urea; 1% ampholines (0,8%. pH 5-7; 0,2%, pH 3-10). Gels

were run at 400 volts for 16h, followed by 800 volts for 1h (total volt-hours = 6 200). Gels were forced out of the tubes using the pressure of a syringe with distilled water. Gels were either prepared for loading in the second dimension immediately or stored frozen in 5ml SDS sample buffer.

Molecular weight separation - 2nd dimension

In this dimension the proteins were separated on a 5-15% polyacrylamide gradient gel (molecular weight range 30-110 x 10^{-3} dalton).

Materials and Methods

T A B L E 10.1.

INGREDIENTS OF 5% - 15% GRADIENT GEL

REAGENT	5%	15%
Acrylogel	2,7ml	8ml
1,5M Tris, pH8,8 with 5% glycerol	4ml	-
1,5M Tris, pH8,8 with 60% glycerol	-	4ml
10% SDS	0,2ml	0,2ml
Water up to:	16ml	16ml
Ammonium persulphate	66µl	53µl
Temed	6µl	6µl

A 5-15% gradient gel (see Table 10.1) was poured from a gradient mixer between glass plates (Hoefer). The gel was overlayed with water and allowed to polymerise for 40 min. The water was then poured off and the gel first overlayed with stacking gel (1,5ml acrylogel, 2,5ml stacking

gel buffer comprising 0,5M Tris, pH6,8 with 5% glycerol, 0,1ml 10% SDS, 40µl ammonium persulphate (100mg/ml), 5µl Temed, made up to 10ml with water) and then overlayed with water. The gel was allowed to polymerise for approximately 20 min, the water removed and the glass sandwiches assembled into the upper tank. The rod gels were briefly immersed in 10ml heated SDS sample buffer (10% w/v glycerol, 2,3% SDS, 0,0625M Tris/pH 6,8, 5% β mercaptoethanol). Excess buffer was removed and the rod gels embedded on top of the sandwiches with 0,1% agarose preheated to 80°C. The agarose was allowed to set, the lower and upper tanks filled with tank buffer (12g Tris, 57,6g glycine and 40ml 10% SDS in 4 l of water) and the gels electrophoresed at 20mA per gel for 10h.

Staining

The gel plates were prised apart and the gel stained with Coomassie blue (5g Coomassie blue stain, 1,5ml methanol, 3,1ml water and 500g trichloroacetic acid) for 6h and then destained for up to 72h. Silver staining was performed according to the technique of Guevara et al., 1982.

Standardisation

Standardisation of the gels was initially obtained by co-electrophoresing the proteins with a set of standards of known molecular mass and isoelectric point.

Analysis of gels

The intensity, size, position and number of protein spots were assessed by visual inspection. With the use of grid lines each gel was divided into 25 squares as shown in Fig. 10.1 which ensured systematic analysis. Reference to specific spots is possible by employing a notation system based on that of Bravo and Celis (1982). This was not applied in this case, but reference spots were identified.

Fig. 10.1.

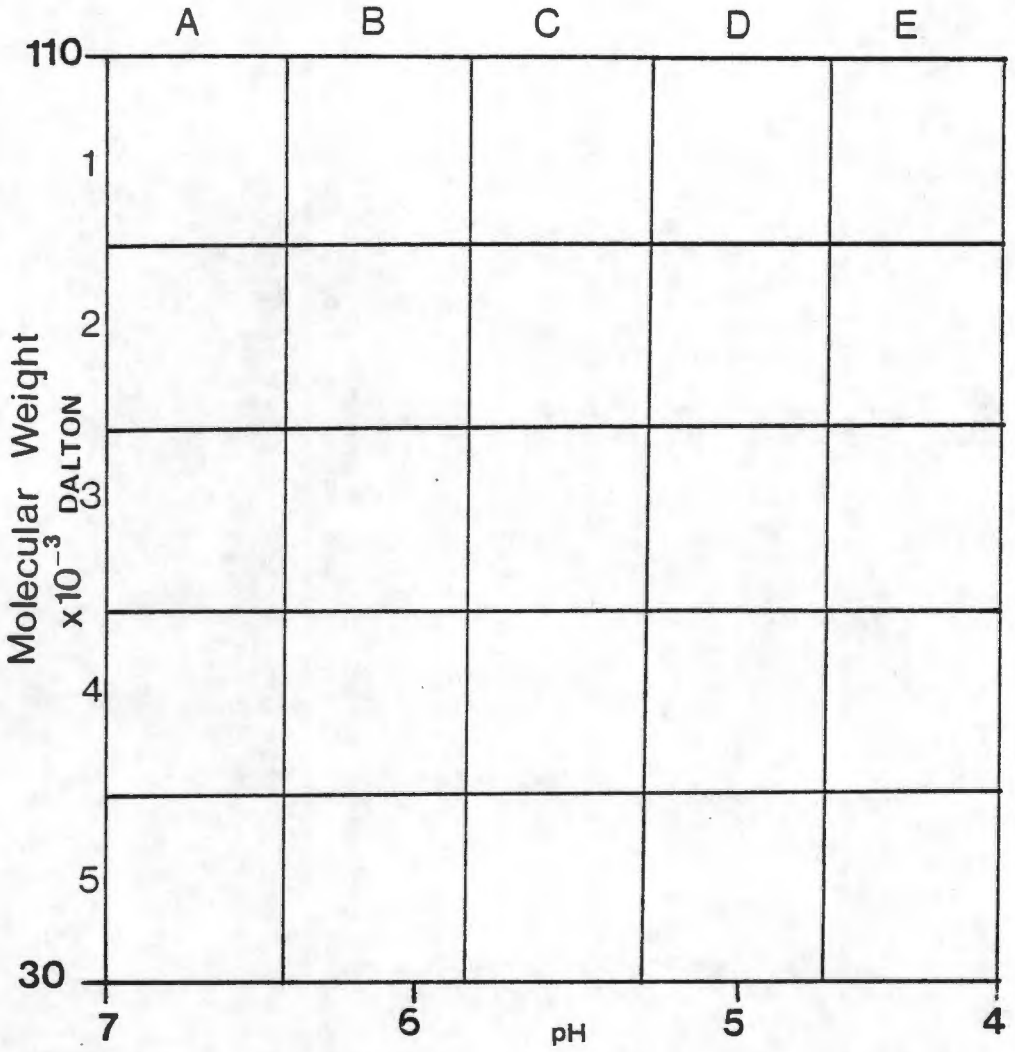


DIAGRAM TO SHOW THE USE OF GRID LINES TO DIVIDE THE GEL INTO 25 SQUARES.

10.3. RESULTS

From gels stained with Coomassie blue approximately 450 discrete spots were identified (Figs. 10.2 and 10.3). Silver staining increased the number of spots by ±20% (Figs. 10.4 and 10.5). Definition and discrimination were good and there was more than 95% reproducibility of gel patterns. Table 10.2 gives the number of gels analysed after maternal administration of 10 000 and 12 500 IU vitamin A on day 9 p.c. in 11-day and 18-day fetuses. Thirty-four gels were stained with Coomassie blue and 6 were silver stained.

TABLE 10.2.

THE NUMBER OF 2-D GELS ANALYSED IN 11-DAY AND 18-DAY FETUSES

DOSE OF VITAMIN A (IU)	NUMBER OF GELS (Sacrifice day 11 p.c.)	(Sacrifice day 18 p.c.)
0 (control)	12	10
10 000	6	4
12 500	4	6

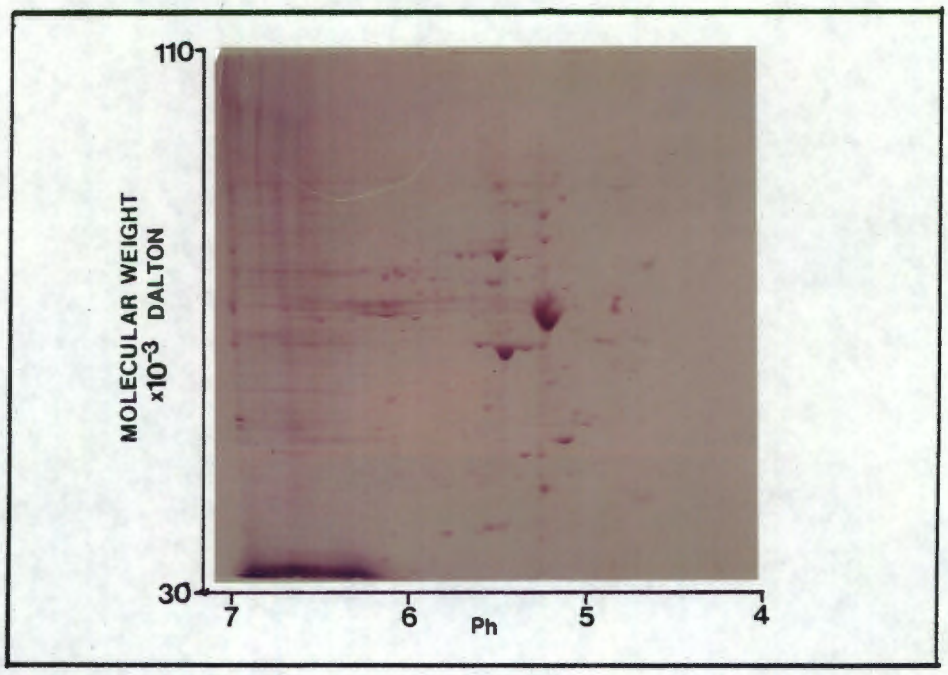
There was no obvious difference in the cephalic protein patterns of untreated 11-day and 18-day fetuses.

A broad spectrum of deletion of 10 - 15% of protein spots was noted and a limited number of new protein spots appeared in the B2, C2 and D3 sectors of vitamin A treated fetuses compared with controls.

10.4. DISCUSSION

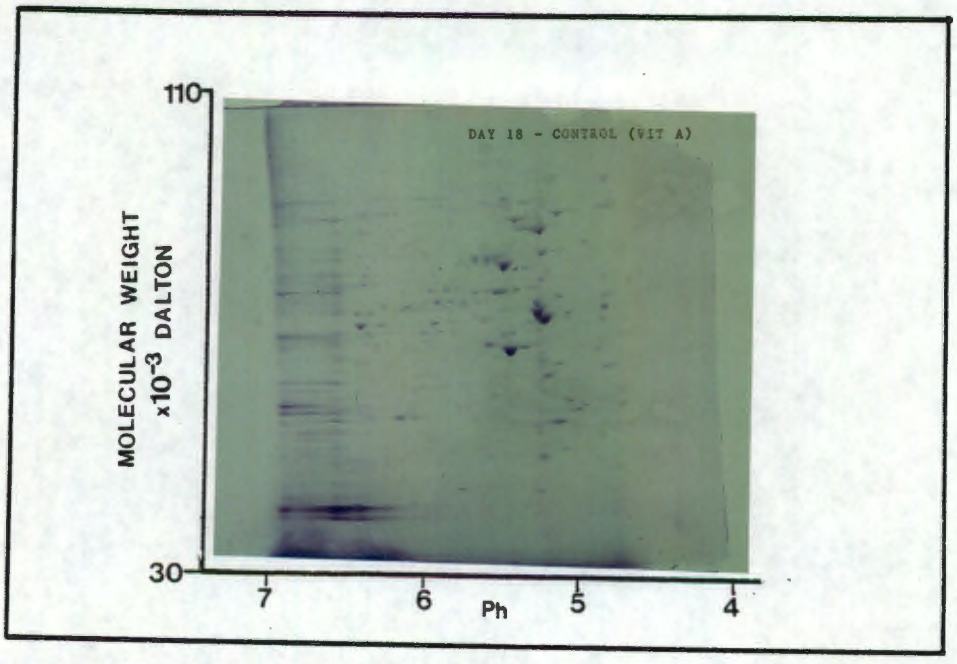
The broad spectrum deletion of protein spots in this study suggests that teratogenic doses of vitamin A modify the brain protein pattern of the fetal C3H mouse in a non-specific way. The new protein spots observed may suggest induction of proteins. Heat-shock-induced proteins have been detected in human cells (Anderson et al., 1982, and in rat embryo fibroblasts (Welch, 1985). However, further experience with and refinement of this technique is needed before meaningful conclusions can be drawn. The number of protein spots which were visualised is relatively small for brain tissue. Celis and Bravo (1981) were able to identify approximately 1 200 spots from as few as 50-100 He La cells by using 35 S methionine labelling conditions. It is envisaged that radiolabelling

Fig. 10.2



COOMASSIE BLUE STAIN GEL ELECTROPHORETIC PROTEIN PATTERN
OF 11-DAY FETAL MOUSE BRAIN (CONTROL)

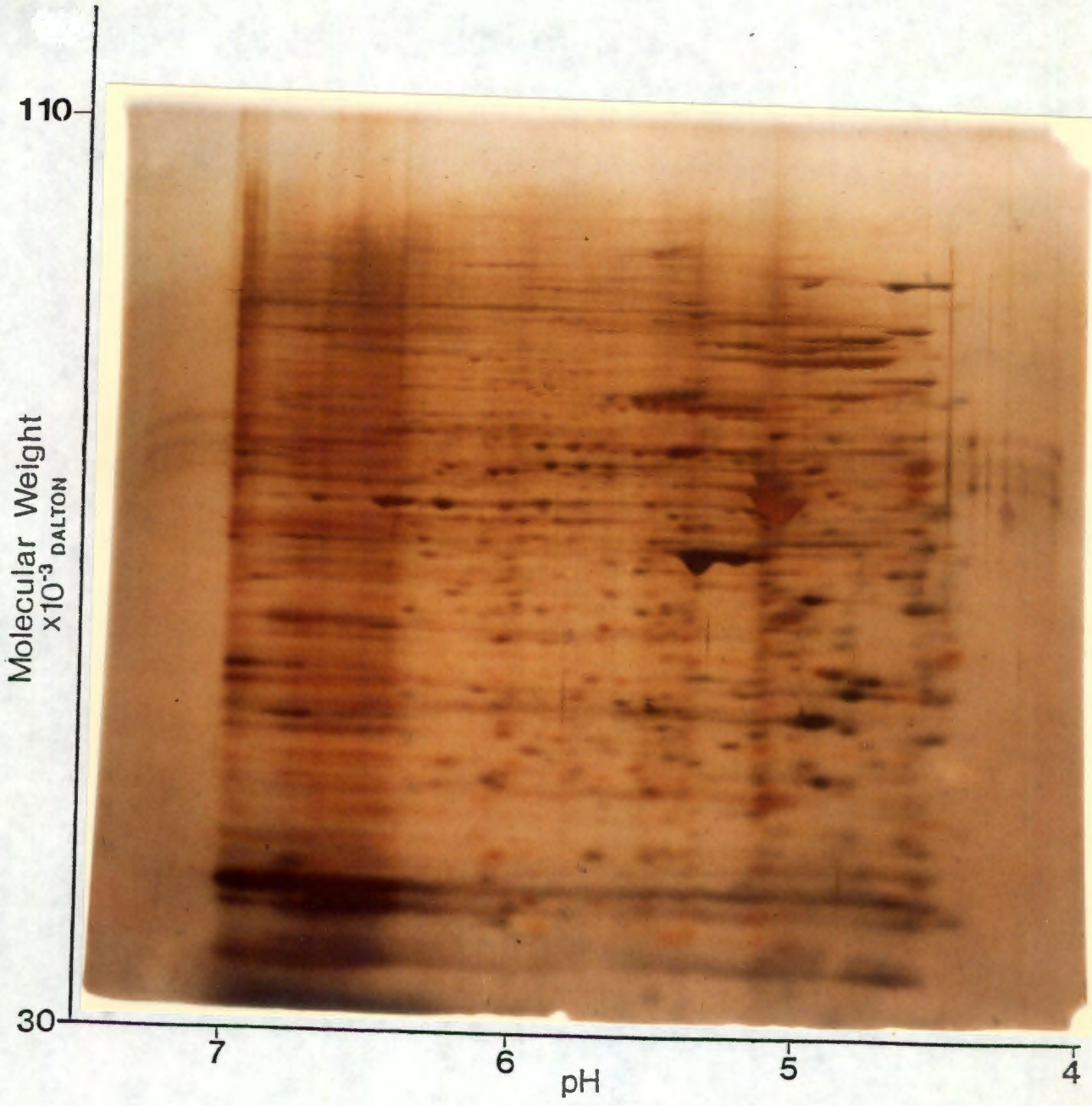
Fig. 10.3



COOMASSIE BLUE STAIN GEL ELECTROPHORETIC PROTEIN PATTERN
OF 18-DAY FETAL MOUSE BRAIN (CONTROL)

Note: Approximately 323 spots were counted in each of the above gels.

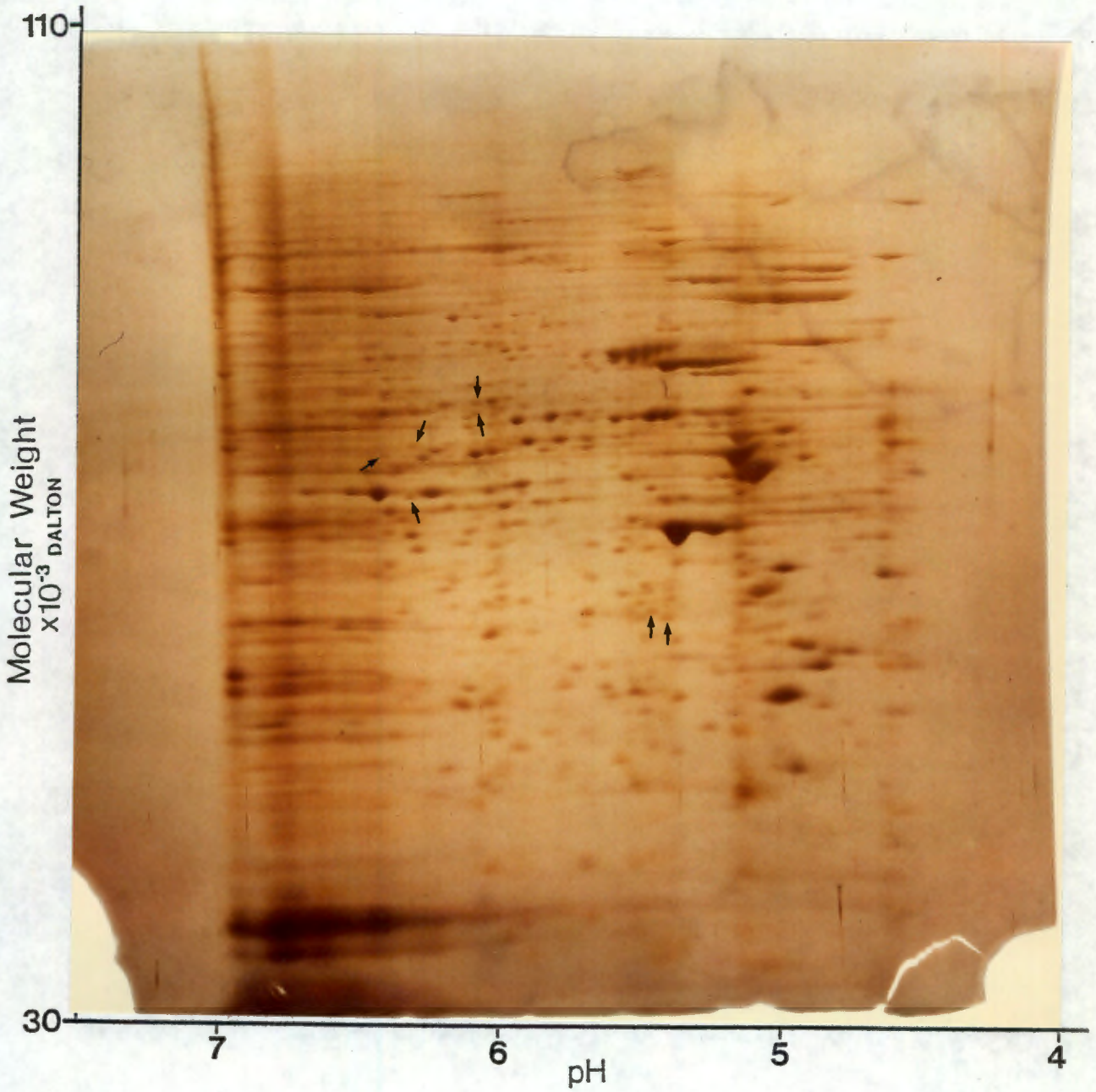
Fig. 10.4.



SILVER STAIN GEL ELECTROPHORETIC PROTEIN PATTERN OF 11-DAY FETAL MOUSE BRAIN (CONTROL)

Note: The number of spots counted in this gel was 458.

Fig. 10.5.



SILVER STAIN GEL ELETROPHORETIC PROTEIN PATTERN OF 11-DAY FETAL MOUSE BRAIN AFTER MATERNAL TREATMENT WITH 10 000 IU VITAMIN A ON DAY 9 p.c.

Note:
i) The broad spectrum deletion of protein spots (compare Fig. 10.4).
ii) Major spots are unchanged.
iii) New protein spots (arrowed) in sectors B2, C2 and D3.
iv) The number of spots counted in this gel was 465.

of proteins and subsequent radiography will augment the sensitivity of this method. Another technique for increasing the number of spots is the use of larger gels (Klose et al., 1977). More spots become visible because the resolving power is improved and the intensity of weak spots is increased due to the larger amounts of protein samples used. The use of different pH ranges will also enable the visualisation of proteins which do not run only in the range 4 - 7.

A further limitation of the present system is that visual inspection is only semiquantitative making subtle quantitative changes impossible to detect by eye (Ridder et al., 1984). Also, information overload is quickly experienced with a large number of spots and when more than 2 gels must be compared. Ridder et al., (1984) have described an image-analysis technique to determine spot intensities and to match spot patterns among gels. An alternative approach is a computer-assisted densitometry technique for quantifying individual protein spots (Goldman et al., 1982; Narayan et al., 1984). Anderson et al., (1984) have used multivariate statistical analysis of computer-generated quantitative data from 2D gels. Computer programs also facilitate comparison between the pattern of spots seen on different gels (Alexander et al., 1980; Skolnick et al., 1982). In fact it has been suggested that 2D electrophoretic separation of complex mixtures of proteins can only be exploited to its fullest potential using sophisticated computerised spot detection, quantification, pattern recognition, pattern normalisation, data reduction and data storage (Gersten et al., 1983)

After appropriate attention to the foregoing further experiments with vitamin A are envisaged, including exposure on other days of gestation.

10.5. CONCLUSIONS

1. The non-specific deletion of protein spots and the apparent induction of new proteins in this study suggest that teratogenic doses of vitamin A modify the brain protein pattern of the fetal C3H mouse.

2. This technique has the potential to develop a test system aimed at detection of newly induced point mutations in fetuses exposed to teratogenic agents and it might give an indication of underlying mechanisms. This potential was not realised in the study reported here, for the reasons that are likely to include those that have been provided.

CHAPTER 11

AN IN VIVO STUDY OF THE EFFECTS OF CYCLOPHOSPHAMIDE, AND LARGE DOSES OF VITAMIN A ON CEPHALIC DNA DAMAGE IN THE FETAL MOUSE USING A DNA UNWINDING ASSAY TO DETECT DNA STRAND BREAKS

11.1. INTRODUCTION

The critical target in terms of the antineoplastic, carcinogenic and mutagenic properties of cyclophosphamide is DNA. As discussed in section 1.2, although the mechanism of cyclophosphamide teratogenicity is unknown, available evidence supports the hypothisis that DNA is the primary target (Mirkes, 1985). Cyclophosphamide metabolites have been shown to alkylate embryonic DNA (Murthy et al., 1973), to inhibit DNA synthesis (Short et al., 1972), and monofunctional phosphoramide mustard has been found to induce fetal DNA strand breaks (Mirkes et al., 1984).

It appears less likely that DNA damage plays an important part in vitamin A teratogenicity. Although the vitamin has been noted by some workers to decrease fetal DNA synthesis (Dingle, Lucy and Fell, 1961; Langman and Welch, 1967; Nanda, 1971; Morriss, 1972; and Kochar, 1975), Kochar (1975) was unable to show an effect on DNA synthesis with concentrations of vitamin A ten times that which cause malformations in culture. Similarly, Eckhert and Hurley (1979) could not demonstrate an effect on DNA synthesis in rat fetuses exoposed to doses of vitamin A which had induced a 61% incidence of malformations.

The aim of the present study was:

i) To compare the effects of cyclophosphamide and vitamin A on cephalic DNA damage in the C3H mouse fetus;

ii) To utilise an in vivo technique which is rapid and simple,

without the necessity of the usual requirements of culturing or radiolabelling;

iii) To elucidate the teratogenic mechanisms of cyclophosphamide and vitamin A with particular reference to the CNS.

A fluorometric method for rapid detection of DNA strand breaks was used (Birnboim and Jevcak, 1981). DNA strand breaks can be detected with great sensitivity by methods which utilize the observation that the rate of unwinding of the 2 DNA strands in alkali is related to the covalent length of strands (Kohn and Ewig, 1973; Rydberg, 1975). DNA strand breaks are associated with increased unwinding which was monitored with the fluorescent dye, ethidium bromide.

11.2. MATERIALS AND METHODS

11.2.1. Experimental mice

The housing of mice and method of mating have been described in Chapter 2.

11.2.2. Drug administration

Vitamin A, 30 000 or 60 000 IU, was administered by gastric intubation on day 11 p.c. to pregnant C3H mice. An equal volume of arachis oil was administered at the same time to controls.

Cyclophosphamide, 15, 30 or 60mg/kg, was administered s.c. on day 11 p.c. to pregnant C3H mice. An equal volume of distilled water was administered at the same time to controls.

11.2.3. Sacrifice, fetal decapitation and head storage

Treated and control mice were sacrificed by cervical dislocation from 6 -

40h after drug administration. The abdominal cavity was opened and uterine horns externalised. The number of fetuses was noted, and fetuses removed and decapitated by a horizontal incision in line with the bottom of the lower jaw. Heads were then stored at -20°C until assayed.

11.2.4. DNA unwinding assay for detection of DNA damage

The principle of the procedure is as follows:

The fluorescent dye, ethidium bromide, binds selectively to double-stranded DNA in the presence of single-stranded DNA when short duplex regions in single-stranded DNA molecules are destablized by alkali (Morgan and Pullyblank, 1974). Conditions were used under which the dye fluoresces preferentially with double-stranded DNA. The cephalic cell lysate was divided equally among 3 sets of tubes. The contribution to fluorescence by components other than double-stranded DNA (including free dye) was estimated from a blank sample (B) treated under conditions which caused complete unwinding of DNA. A second sample was used for estimating total fluorescence (T) i.e. fluorescence due to the presence of double-stranded DNA plus contaminants. The difference (T-B) provided an estimate of the amount of double-stranded DNA in the extracts. A third sample (P) was used to estimate the degree of unwinding of the DNA. The cephalic lysate was exposed to alkaline conditions sufficient to permit partial unwinding of the DNA. The fluorescence of this sample less the fluorescence of the blank (P-B) provided an estimate of the amount of double-stranded DNA remaining.

Heads were placed individually in microfuge tubes at 4°C and 300µl of phosphate buffered saline (PBS) added. Disruption of cells was gently initiated using a pipette. Nine hundred microlitres of solution A, (0,87% NH_4Cl, 10mM Tris-HCl, pH7,2) was added and the suspension left until cell lysis was complete (20 - 30min). The suspension was centri-

fuged at 12 000g for 1min, the supernatant discarded and the pellet resuspended in 1ml of solution A, then centrifuged at 12 000g for a further 1min. This pellet was resuspended in 0,8ml of solution B (0,25M meso-inositol, 10mM sodium phosphate, 1mM $MgCl_2$, pH7,2) to give a cell count of 5 - 10 x 10^6/ml). Two hundred microlitre aliquots of this suspension were dispensed into glass tubes designated T, P and B. To each was added 200µl of solution C (9M urea, 10mM NaOH, 2,5mM cyclohexanediamine-tetraacetate, 0,1% sodium dodecyl sulphate), and incubated at 4°C for 10min (during this time chromatin disruption occurs).

To the P and B tubes, 100µl of solution D (0,45 volume solution C in 200mM NaOH) and 100µl of solution E (0,4 volume solution C in 200mM NaOH) were gently added without mixing at 4°C for 30min. The contents of the B tube were sonicated for 2sec to ensure rapid denaturation of the DNA in alkaline solution. P and B tubes were incubated at 15°C for 60 min. Denaturation was stopped by addition of 400µl of a solution containing 1M glucose and 14mM mercaptoethanol (solution F) at 0°C, which lowered the pH to $\pm$ 11. One hundred microlitres of solution D and E were added to the tube T at 0°C. All 3 tubes were sonicated briefly to give a homogeneous suspension and diluted with 1,5ml of ethidium bromide (6,7µg/ml, 13,3mM NaOH). Fluorescence was read at room temperature in a Perkin-Elmer luminescence spectrometer (excitation 520nM; emission 590nM).

The T tubes differed from the P tubes in that the neutralising solution, solution F, was added before the alkaline solutions D and E so that the DNA was never exposed to a denaturing pH.

The extent of DNA unwinding after a given time of exposure of cephalic cell lysates to alkali was calculated from the fluorescent values of the T, P and B samples. The percent of double-stranded DNA remaining after partial alkali treatment was given by (P-B)/(T-B) x 100. Subtracting this

value from that obtained for controls gave the percentage conversion of double-stranded DNA to the single-stranded form. For example, if the percentage of double-stranded DNA for controls was 75% and that of the treated fetuses 52%, then 23% of the DNA in the treated fetuses was converted to the single-stranded form.

The reagents described give a final pH value which is high enough to destablilize single-stranded DNA and RNA (to give low B fluorescence values) yet still below the denaturing pH for double-stranded DNA (Birnboim and Jevcak, 1981).

11.2.5. Statistical analysis

The Mann-Whitney U two-tailed test was used to compare data from treated fetuses with control data. Medians and 95% confidence limits were calculated from the Walsh averages, after Tukey (Steinijans and Diletti, 1983).

11.3. RESULTS

11.3.1. The effects of cyclophosphamide on cephalic DNA damage

The effect of the dose of cyclophosphamide and the time of assay after administration an murine fetal cephalic DNA damage is shown in Table 11.1 , where DNA damage is expressed as the percentage of double stranded DNA.

The time course of cephalic DNA damage is shown in Fig. 11.1 , where DNA damage is expressed as the percentage conversion of DNA to the single-stranded form.

The effect of incremental doses of cyclophosphamide on cephalic DNA damage 16h after administration is shown in Fig. 11.2 (expressed as the percentage

T A B L E 11.1.

THE EFFECT OF THE DOSE OF CYCLOPHOSPHAMIDE AND THE TIME OF ASSAY AFTER ADMINISTRATION ON MURINE CEPHALIC DNA DAMAGE

DOSE (mg/kg)	TIME (h) AFTER ADMINISTRATION OF ASSAY					
	3	6	9	16	22	30-40
0	78% (66-91) n=6	80% (74-87) n=13	86% (75-95) n=6	75% (72-80) n=47	71% (63-89) n=12	78% (68-87) n=20
15	72% † (68-76) n=7	45% * (38-50) n=11	37% * (33-40) n=5	52% * (47-57) n=33	83% † (73-89) n=14	76% † (64-88) n=24
30	75% † (55-87) n=6	24% * (20-29) n=12	30% * (25-30) n=6	45% * (40-50) n=42	78% † (65-90) n=11	68% † (65-82) n=5
60	-	-	-	31% * (25-40) n=34	-	-

DNA DAMAGE EXPRESSED AS THE PERCENTAGE OF DOUBLE STRANDED DNA.

n = no of assays (= no of fetal heads)

* $p<0,0003$

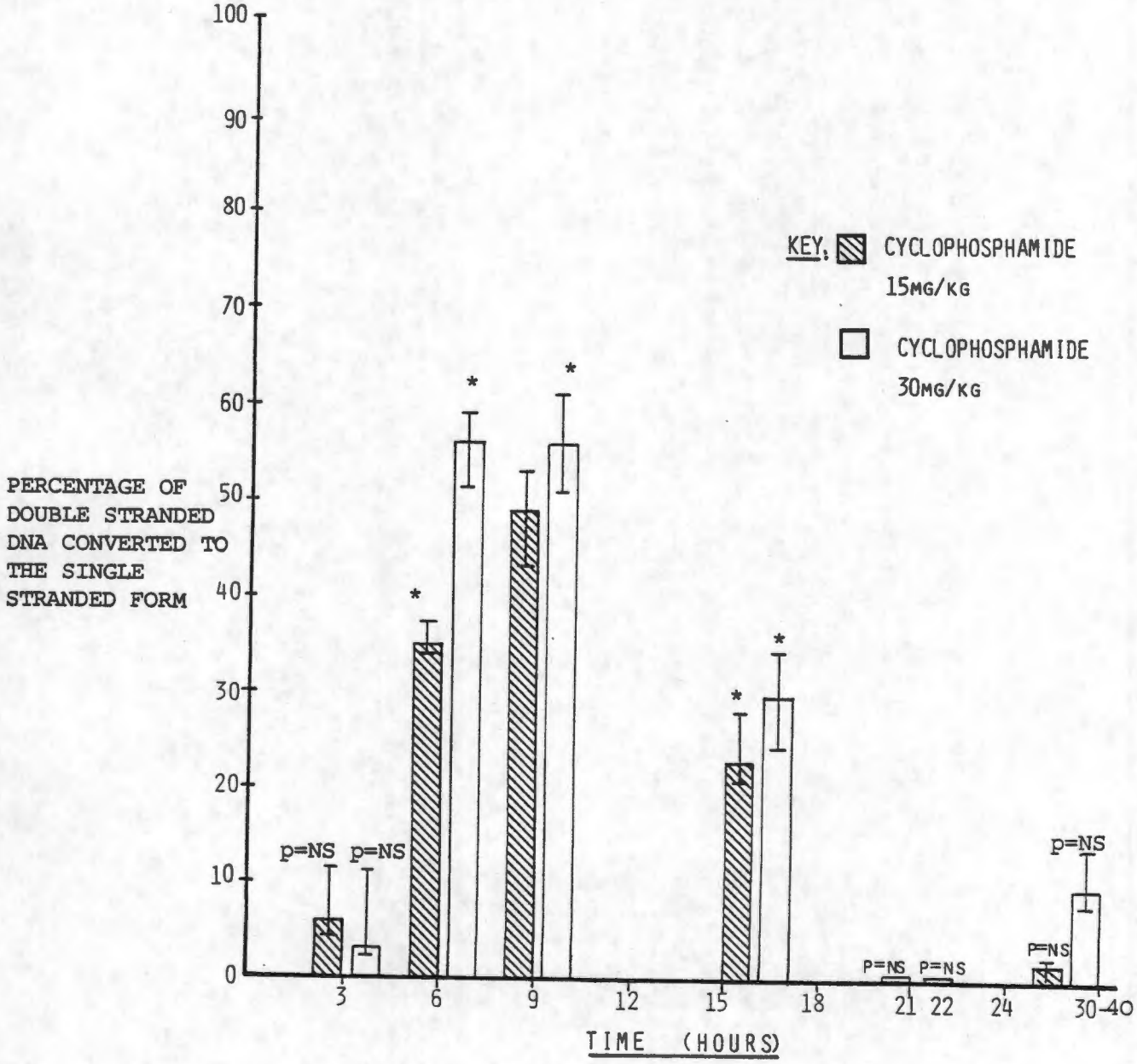
† p = NS

p value determined by comparison with control data using Mann-Whitney U test.

Figures in brackets are the 95% confidence range.

Fig. 11.1.

TIME COURSE OF CEPHALIC DNA DAMAGE INDUCED BY CYCLOPHOSPHAMIDE.



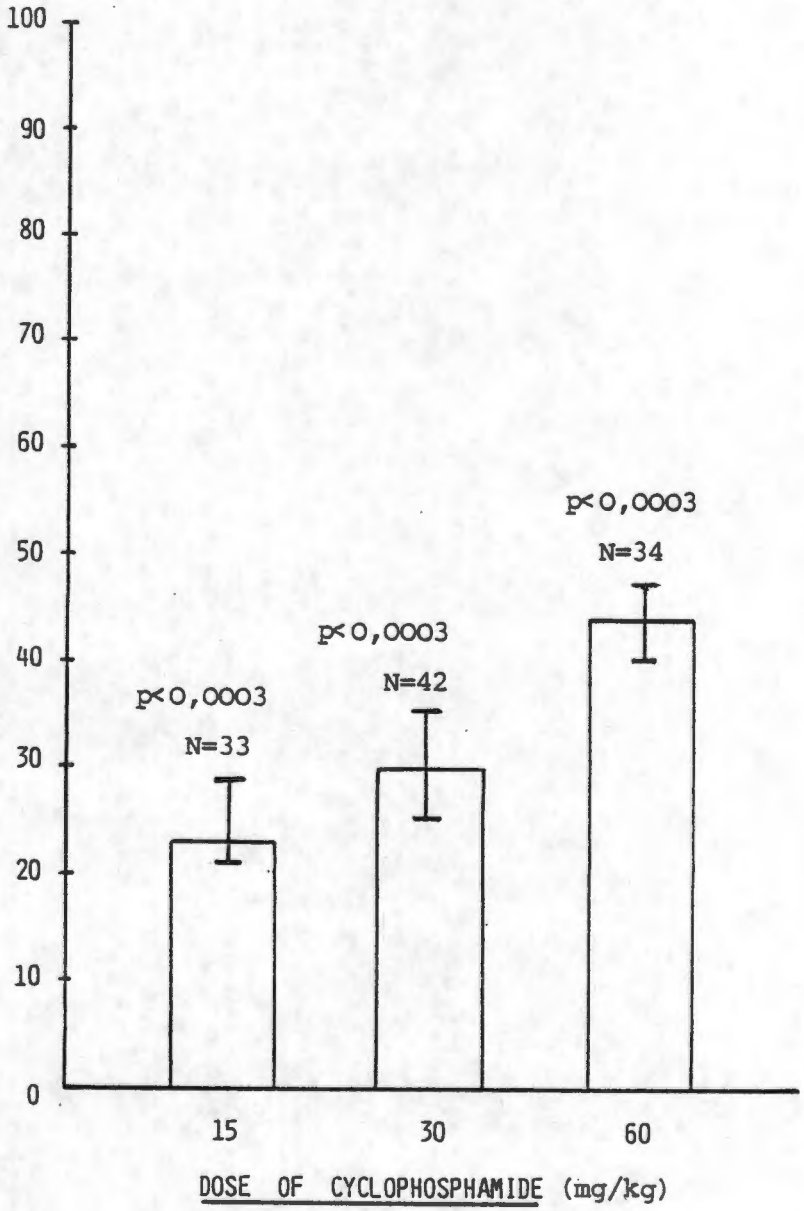
DNA DAMAGE EXPRESSED AS % CONVERSTION OF DNA TO THE SINGLE STRANDED FORM

* $p<0,0003$

p value determined by comparison with control data using Mann-Whitney U test.

Fig. 11.2.

THE EFFECT OF INCREMENTAL DOSES OF CYCLOPHOSPHAMIDE ON MURINE FETAL CEPHALIC DNA DAMAGE 16h AFTER ADMINISTRATION EXPRESSED AS THE PERCENTAGE CONVERSION OF DNA TO THE SINGLE-STRANDED FORM



p value determined by comparison with control data using Mann-Whitney U test.

conversion of DNA to the single-stranded form).

There was no evidence of cephalic DNA damage in 12-day fetal mice at 3h after administration (the median percentage of double-stranded DNA of untreated mice was 78% compared with 72% and 75% in fetuses exposed to cyclophosphamide 15mg/kg and 30mg/kg respectively). However, at 6h after cyclophosphamide administration there was obvious evidence of DNA damage. The percentage conversion of DNA to the single-stranded form was 35% and 56% for fetuses exposed to 15mg/kg and 30mg/kg respectively, $p<0,003$. Similarly, evidence of DNA strand breaks was present and still maximal at 9h (49% and 56% conversion in fetuses exposed to 15mg/mg and 30mg/kg cyclophosphamide respectively, $p<0,003$). At 16h, although there was significant DNA damage, as can be seen in Fig. 11.1, it appeared to be less than at 6h and 9h (23% and 30% conversion in fetuses exposed to 15mg/kg and 30mg/kg, respectively).

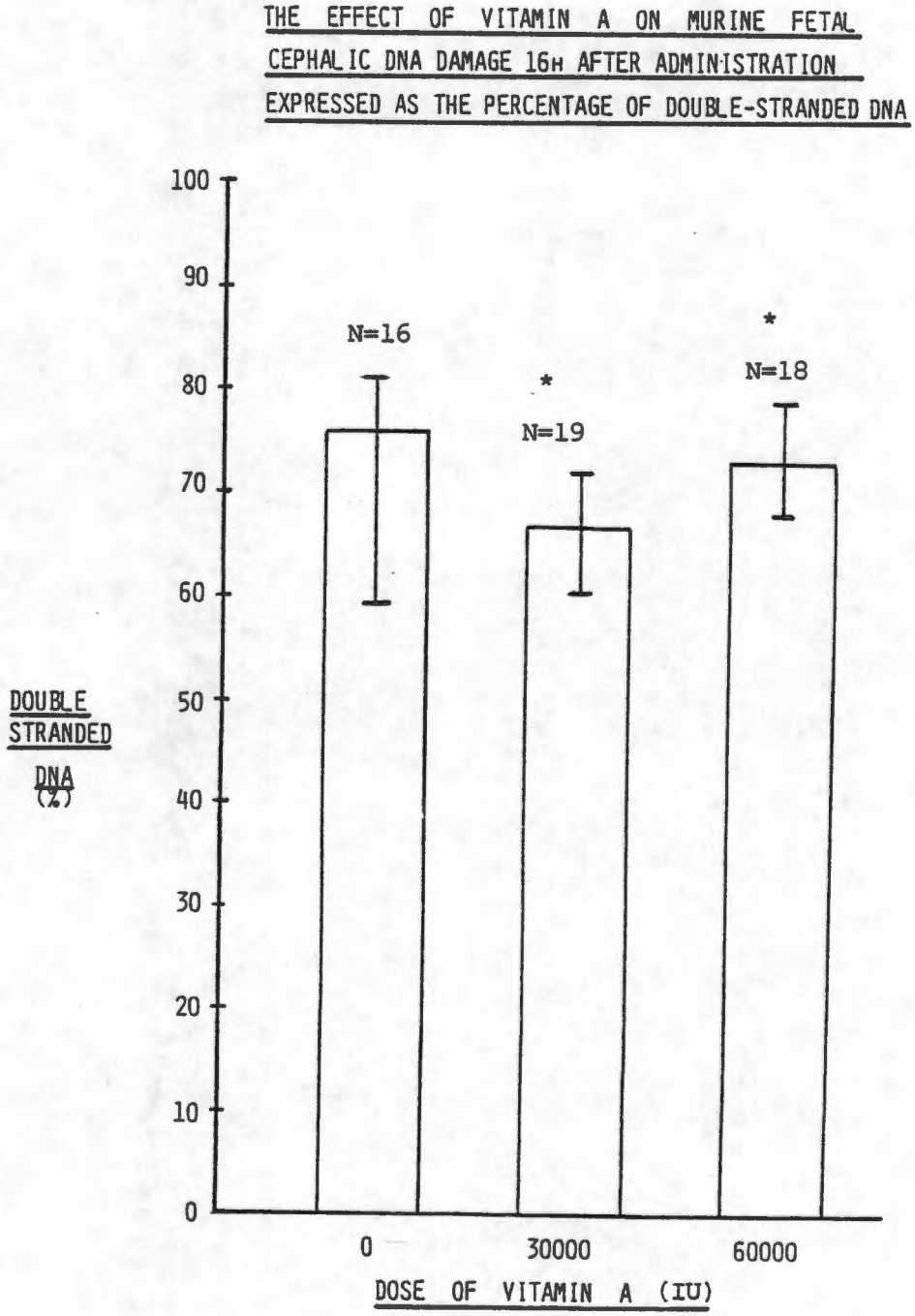
At 22h and 30-40h after cyclophosphamide there was no evidence of DNA strand breaks.

An increase in DNA damage was evident in fetal heads examined 6, 9 and 16h after cyclophosphamide administration (see Fig. 11.1). Fig. 11.2 further illustrates the effect of incremental doses (15, 30 and 60mg/kg) 16h after cyclophosphamide administration, suggesting a dose-related increase in cephalic DNA damage.

11.3.2. <u>The effect of vitamin A on murine fetal cephalic DNA</u>

Teratogenic doses of 30 000 and 60 000 IU vitamin A administered on the 11th day post conception were not associated with any evidence of DNA strand breaks. The percentage of double-stranded DNA in untreated fetuses was 76% compared with 67% and 73% in fetuses exposed to 30 000 and 60 000 IU respectively - see Fig. 11.3. These doses of vitamin A administered

Fig. 11.3.



* p = NS

p value determined by comparison with control data using Mann-Whitney U test.

on day 11 p.c. were obviously teratogenic as shown in the results of an experiment where fetuses were examined on day 18 p.c. (Tables 11.2 and 11.3). There was a 93% incidence of early intra-uterine deaths and one live abnormal fetus after administration of 30 000 IU, and 100% embryolethality with 60 000 IU. (At the time of sacrifice on day 12 in the DNA experiments, fetuses exposed to vitamin A were still viable with normal heartbeats).

11.4. DISCUSSION

The absence of evidence of fetal cephalic DNA damage 3h after cyclophosphamide administration is explicable on the basis of the time needed for maternal absorption, metabolism to active metabolites (Greenaway *et al.*, 1982; Hales, 1983), and distribution via the placenta to the fetus. However, evidence of maximal DNA damage was apparent 3h later at 6h after administration, indicating the rapidity with which marked DNA damage occurs in this *in vivo* model.

In histological studies cell necrosis was only noted in the fetal rat brain 24h after administration of cyclophosphamide (Wendler, 1978), whereas in embryonic limb buds evidence of excess necrosis was apparent 5h after exposure (Manson *et al.*, 1982). The rapid onset in the latter *in vitro* study is not strictly comparable because the exclusion of maternal factors would ensure almost immediate access of the teratogen to the fetal tissues.

Although there was significant DNA damage at 16h, it was less than at 6h and 9h (30% conversion of the single-stranded form of DNA at 16h compared with 56% at 6 and 9h after a dose of 30mg of cyclophosphamide). This was in keeping with the onset of DNA repair and indicates how rapidly this process occurred. By 22h there was no evidence of DNA strand breaks

TABLE NO. 11.2

VITAMIN A, 30 000 IU PER MOUSE, ADMINISTERED ON DAY 11 p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18	Healthy		3 haemorrhagic ± 4mm necrotic fetal remnants with well developed placentae	-
				3 macerated fetuses (± 5mm) with hydramnios	
			1	small live fetus with phokomelia and only 2 toes on each forelimb	
2	18	Healthy		7 haemorrhagic ± 3mm necrotic fetal remnants with well developed haemorrhagic placentae	-

TABLE NO. 11.3

VITAMIN A, 60 000 IU PER MOUSE, ADMINISTERED ON DAY 11 p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18	Wasted, scrawny, hair thinning		8 necrotic fetal remnants (3-4mm) with well developed placentae No live fetuses	-
2	18	Wasted, scrawny, hair thinning		7 necrotic fetal remnants as above No live fetuses	-

demonstrating repair was complete by this stage. In published histological studies of the embryotoxic effects of cyclophosphamide such rapid onset of repair has not been described. Von Kreybig and Schmidt (1967) recorded repair in the course of the second 24h after administration in the fetal rat brain, whereas Wendler noted evidence of repair after 72h (Wendler, 1979). In experiments with 5-fluorodeoxyuridine which blocks DNA replication, repair was evident 24h after application (Langman et al., 1980). It would therefore appear that DNA damage and repair antedate evidence of histological necrosis and repair. The entity of DNA repair is well known (Manson, 1981b), but the rapidity with which it occurs is an interesting finding.

Manson et al., (1982) also attempted to correlate cell death with DNA damage. A trend towards increasing DNA damage with time was observed, the greatest damage occurring during the postnecrotic period at 48 and 72h. These in vitro observations are clearly different from the present study and are not readily explicable, because a decrease in DNA damage would be anticipated during the postnecrotic repair period, such as was found in these experiments.

Because the technique used detected DNA strand breaks this study demonstrates that this primary lesion of DNA is probably important in producing embryotoxic effects, as was reported by Mirkes et al., (1984) where the defects produced by monofunctional phosphoramide mustard were associated with the induction of DNA strand breaks.

The findings in this study are in agreement with the hypothesis that the underlying mechanism of cyclophosphamide induced teratogenicity is DNA damage. Thereafter the pathogenesis of developmental defects could follow the pathway outlined in Fig. 1.2 (section 1.1). Mirkes (1985) suggests

that DNA lesions lead to cell death or mutations ultimately resulting in the final defect.

The absence of any evidence of DNA strand breaks induced by embryotoxic doses of vitamin A in the present study is further support that DNA damage does not play an important part in vitamin A induced teratogenicity. The fetuses in this experiment were examined 16h after administration of vitamin A and it is possible that DNA damage may occur after this period. However, considering the teratogenic dose administered and lack of evidence to the contrary this was considered unlikely and was not pursued.

Although, as outlined in section 11.1, conflicting results have been found by different workers as regards the effect of the vitamin on DNA synthesis, this to my knowledge, is the first study investigating DNA damage.

11.5. CONCLUSIONS

1) The administration of teratogenic doses of cyclophosphamide on the 11th day post conception was associated with an increase in DNA damage in fetal heads examined 16h after dosing, which appeared to be dose-related.

2) There was no evidence of DNA damage 3h after administration, but damage was apparent at 6h and was still present at 16h. However, the absence of DNA damage in fetuses examined at 22, 30 and 40h suggest that DNA repair had occurred.

3) Teratogenic doses of vitamin A administered on the 11th day post conception were not associated with any evidence of DNA strand breaks.

4) The technique is a sensitive and simple one for the in vivo determination of DNA damage induced by teratogens.

CHAPTER 12

CONCLUDING CHAPTER

I have documented substantial AChE activity in 17-day to 19-day C3H fetal mouse brains, and a lower activity of approximately one third in 11- and 12-day fetal heads. However, fetal brain cholinesterase activity was very low, contributing only 3,0 - 6,7% to the total enzyme activity (AChE and ChE), and was not demonstrable on polyacrylamide gel electrophoresis. Five distinct isoenzyme zones of AChE were demonstrated and the band pattern was similar in 17-, 18- and 19-day fetuses.

An observation well known in teratology was the substantial difference in severity and frequency of congenital defects from litter to litter as well as from offspring to offspring within the same litter after administration of all 3 teratogens used in this thesis. No maternal toxicity was observed with the doses of the drugs used in the definitive experiments which lends support to the concept that the fetal abnormalities observed were due to direct effects of the teratogens on the embryo. Other similarities were the rise in incidence of malformations and embryolethality with increase in dose as well as a steep rise in resorption rate after administration earlier in gestation. A steep dose-response curve for malformations was apparent. A striking abnormality was exophthalmos which was most frequently observed after vitamin A administration and appeared to be due to the reduction in size of the orbit. Although a growth-inhibitory effect was demonstrable with all 3 teratogens, it was most pronounced after cyclophosphamide administration and appeared to be dose-related with the dose-response curve to the left of that for malformations and embryolethality. In contrast, the vitamin A-induced growth inhibitory dose-effect curve appeared to be to

the right of the malformations curve.

Because embryotoxic mechanisms vary with the teratogen used and the time of exposure it is not surprising that differences were found in fetal brain AChE activity. The increase in brain AChE activity observed after vitamin A administration was most reasonably explained on the basis of relative sparing of cholinergic neurons from the embryotoxic effects of the teratogen. The absence of an increase in brain total protein content suggested that the greater enzyme activity was not secondary to a general increase in brain protein, and the absence of an increase in ChAT activity pointed against exuberant repair. The decrease in fetal brain AChE (and ChAT) activity after cyclophosphamide and sodium valproate administration was explicable on the basis of a growth-inhibitory effect. In fetuses examined two days after cyclophosphamide administration the greater head AChE activity along with an increased haemoglobin and decreased ChAT activity suggested that the increase was due to blood. This was compatible with a haemorrhagic process and was not a feature of teratogenic injury with vitamin A. The differential effect of the teratogens used on the isoenzyme zones provide evidence that these are indeed isoenzymes and not aggregates, and furthermore suggest distinct embryotoxic mechanisms. Further investigation might have been of interest but because AChE offered no particular advantage as a marker of teratogenic injury this was not pursued.

Numerous points emerge regarding the potential of AChE (and ChAT) as a biochemical marker of teratogenic injury in the fetal CNS. It does not appear to be more sensitive than standard teratogenicity tests because changes in activity were not apparent in the absence of evidence of gross effects such as growth retardation, morphological abnormalities or embryo-lethality. Furthermore, a change in brain enzyme activity was not always observed when there was other evidence of teratogenic effects. It would

appear that a number of factors such as growth inhibition, haemorrhagic processes, repair, relative sparing of neurons, time of administration and oppositve changes in different parts of the brain can all influence whether there is ultimately a detectable overall change in brain AChE activity. Also, the finding that the ontogenetically new cerebral cortex, which would be expected to be the most susceptible part of the brain to teratogenic insults, has a low AChE activity is another potential limitation of this enzyme as a brain marker of teratogenic injury. Perhaps a better choice, rather than an enzyme which is merely a component of the cholinergic nervous system, might have been an enzyme involved in one of the more fundamental functions of embryonic cell physiology. However, although AChE alone is not a reliable indicator of embryotoxicity, when viewed together with other parameters such as growth inhibition, repair, malformations and embryolethality the information is useful and the findings have pointed to some of the underlying processes such as haemorrhagic necrosis, relative sparing of cholinergic neurons, growth inhibition and repair.

The potential of other biochemical markers merits further investigation. Detection of metabolic lesions, such as quantifiable deviations in enzymes, particularly in the absence of gross morphological abnormalities would greatly extend the sensitivity of standard teratological procedures. Another enzyme, ornithine decarboxylase, a regulator of macromolecule synthesis, has recently proven useful as a brain biochemical marker for teratologic events (Slotkin et al., 1985).

Other findings in this thesis have contributed to a better understanding of the pathophysiology of vitamin A-induced embryotoxic effects. Although vitamin A has been reported to decrease fetal cell proliferation and inhibit mitosis, no demonstrable effect was observed in blastocyst cell

number or mitotic index, which tends to exclude these as important mechanisms in this model. Furthermore, viability/esterase enzyme activity of the preimplantation embryo was unaffected, suggesting that the striking incidence of abnormalities noted in fetuses near term was not due to embryotoxic effects operative during the predifferentiation period. It is apparent the developmental aberrations were due to effects operating during the post-implantation period, which is consistent with the long half-life of elimination of vitamin A. Despite the apparent resistance to teratogenesis of the pre-differentiation embryo these findings caution against the use of any potentially embryotoxic drug with a long half-life during this period.

The findings in this study also suggest that the production of chromosomal abnormalities is not an underlying mechanism of vitamin A teratogenicity in the mouse. Similarly, the absence of any evidence of DNA strand breaks induced by embryotoxic doses of vitamin A is further support that DNA damage does not play an important part in vitamin A induced teratogenicity. However, the finding that vitamin A altered brain protein patterns in the fetal mouse may be a pointer to an important mechanism whereby vitamin A causes abnormal embryogenisis. High resolution 2D electrophoresis of protein requires further investigation as a potential tool for determining the teratogenic effects and mechanisms of drugs.

The evidence in this thesis for the apparently dose-related fetal cephalic DNA damage induced by cyclophosphamide provide further support for the importance of this mechanism in cyclophosphamide induced teratogenicity. Of particular interest was the rapidity with which this DNA damage occurred in vivo, and likewise the rapidity with which repair occurred. The findings also demonstrate that the induction of DNA strand breaks is important in producing the emrbyotoxic effects of cyclophosphamide. In addition, the technique described is a sensitive and simple one for the in vivo deter-

mination of DNA damage induced by teratogens. Following the teratogenic insult of DNA damage, impaired biosynthesis or mutations may result leading to decreased cellular proliferation and increased cell death, and ultimately abnormal differentiation and morphogenesis causing the final defect such as malformation.

In conclusion, progress has been made towards the accomplishment of the two major objectives of this thesis, namely the definition of the mechanism of action of teratogens, and the development of test methodologies. There is still much work to be done before more sensitive markers of teratogenic injury are found and until there is a thorough understanding of the pathogenic mechanisms of drug injury to the fetus. I feel research directed at defining abnormalities at a biochemical or subcellular level is of primary importance. It is hoped that the findings in this study will stimulate further investigation and will ultimately play a role in decreasing the incidence of drug-related birth defects.

APPENDIX 1

STATISTICAL TESTS USED

Because of the bimodal nature of teratological results non-parametric statistics were used. Similarly, medians and 95% confidence limits were employed rather than means, since the former reflects central tendency of a skewed distribution better than the mean. The difficulty arises in establishing the confidence limits of the median. A useful approach is to use the Tukey modification of Wilcoxon's signed rank matched pairs test, as described by Steinijans and Diletti (1983). Medians and confidence limits are calculated using Walsh averages and the Wilcoxon Sum T^+ of positive ranks. This method of establishing central tendency and scatter for a group of data compares well with parametric equivalents for data where either nonparametric or parametric tests may be used.

The Mann-Whitney U Test (Segal, 1956a), one of the most powerful of the nonparametric tests and a useful alternative to the parametric t test without the t test's assumptions, was used to compare test and control data.

The Chi-square test (Segal, 1956b) was used to determine the significance of the differences among independent groups.

The Kolmogorov-Smirnov two sample test (Segal, 1956c) which is sensitive both to differences in means and in distribution, was used in certain situations where a bimodal population was suspected.

All p values quoted were for 2 tailed tests and a p value of less than 0,05 was regarded as statistically significant.

A P P E N D I X 2

TABLES OF ORIGINAL DATA

Note: Tables are printed on both sides of the page.

For parametric data calculations of means, standard derivations and linear regressions were performed as required. Reference was made to standard texts for derivations.

Calculations were carried out using a Hewlett-Packard desk top calculator model HP9825A.

TABLE NO. 6.2T

TEST ANIMALS

50000 IU VITAMIN A ON DAY 9½ p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Scrawny, hair thinning		No live fetuses (All resorbed)	8
2	18½	Scrawny, hair thinning		No live fetuses (All resorbed)	5
3	18½	Scrawny, hair thinning		No live fetuses (All resorbed)	4
4	18½	Scrawny, hair thinning		No live fetuses (All resorbed)	8

TABLE NO. 6.2C

CONTROLS

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1	Normal	1
			2	Normal	
			3	Normal	
			4	Normal	
			5	Normal	
			6	Normal	
2	18½	Healthy	7	Normal	0
			8	Normal	
			9	Normal	
			10	Normal	
			11	Normal	
			12	Normal	
			13	Normal	
3	18	Healthy		1 macerated IUD	0
			14	Normal	
			15	Normal	
			16	Normal	
			17	Normal	
			18	Normal	
			19	Normal	
			20	Normal	
			21	Normal	

TABLE NO. 6.3T

TEST GROUP

15000 IU VITAMIN A ON DAYS 8½ AND 9½ p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy		No live fetuses (All resorbed)	5
2	18½	Healthy		No live fetuses (All resorbed)	4
3	18½	Healthy		No live fetuses (All resorbed)	8
4	18½	Healthy		No live fetuses (All resorbed)	4
5	18½	Healthy	1	Exophthalmos, hypoplastic face, short tail	6

TABLE NO. 6.3C

CONTROLS

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1 2 3 4	Exomphalos	0
2	18½	Healthy	5 6 7 8 9 10 11	-	0
3	18½	Healthy	12 13 14 15 16 17	-	1

TABLE NO. 6.4T

TEST ANIMALS
15000 IU VITAMIN A ON DAY 9½ p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1	Exophthalmos, abnormal shaped head, absent tail, myelomeningocele	0
			2	Exophthalmos, absent tail	
			3	Exophthalmos, absent tail	
			4	Exophthalmos, absent tail, small	
			5	Exophthalmos, absent tail, small	
			6	Exophthalmos, absent tail, small	
2	18½	Healthy	7	1 macerated IUD monster	3
3	18½	Healthy	8	Normal	2
			9	Normal	
			10	Normal	
			11	Normal	
			12	Normal	

TABLE NO. 6.4T (Contd.)

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
3	18½	Healthy	13	Normal	0
			14	Exophthalmos, abnormal shaped head, kinky tail	
4	18½	Healthy	15	1 macerated IUD Exophthalmos	0
			16	Exophthalmos	
			17	Exophthalmos	
			18	Small	
			19	Small	
5	18½	Healthy	20	Normal	3
			21	Exophthalmos	

TABLE NO. 6.4c

CONTROLS

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy		1 small macerated IUD	0
			1	Normal	
			2	Normal	
			3	Normal	
			4	Normal	
			5	Normal	
			6	Normal	
			7	Exomphalos	
2	18½	Healthy	8	Normal	0
			9	Normal	
			10	Normal	
			11	Normal	
			12	Normal	
			13	Normal	
3	18½	Healthy	14	Normal	0
			15	Normal	
			16	Normal	
			17	Normal	
			18	Normal	

TABLE NO. 6.4C (Contd.)

CONTROLS

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
				1 macerated IUD	
4	$18\frac{1}{2}$	Healthy	19	Normal	1
			20	Normal	
			21	Normal	
			22	Normal	
			23	Normal	
			24	Normal	

TABLE NO. 6.5T

TEST : 10000 IU VITAMIN A ON DAY 9½ p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1 2 3 4	Exophthalmos, absent tail " " " " " " Exophthalmos, absent tail and exomphalos	2
2	18½	Healthy	5 6 7 8 9	Exophthalmos " Exophthalmos and exomphalos	1
3	18½	Healthy	10 11 12 13 14 15 16 17	1 macerated IUD Exophthalmos "	0

TABLE NO. 6.5c

CONTROL

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1 2 3 4 5 6 7		0
2	18½	Healthy	8 9 10 11 12	1 IUD	1
3	18½	Healthy	13 14 15 16 17	1 IUD	0

TABLE NO. 6.6T

TEST : 10000 IU VITAMIN A ON DAY 9½ p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1	Exencephaly, exophthalmos, hydramnios (blood stained)	3
			2	-	
			3	Exencephaly, exophthalmos	
2	18½	Healthy	4	Exophthalmos	3
			5	"	
3	18½	Healthy	6	-	0
			7	-	
			8	Exophthalmos	
			9	-	
			10	-	
			11	-	
			12	-	
			13	-	
4	18½	Healthy	14	-	2
			15	-	
			16	-	
			17	-	
			18	-	

TABLE NO. 6.6T (Contd.) TEST

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
5	18½	Healthy	19	-	0
			20	-	
			21	-	
			22	-	
			23	-	
			24	-	
			25	-	
6	18½	Healthy	26	Fresh IUD, exophthalmos	0
			27	Runt, exophthalmos	
			28	" "	
			29	" "	
			30	" "	
			31	Exophthalmos	
			32	-	

TABLE NO. 6.6C

CONTROL

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1		Healthy	1 2 3	- - Exomphalos	2
2		Healthy	1 2 3 4 5	- - - - -	2
3		Healthy	6 7 8 9 10	- - - - -	2
4		Healthy	11 12	- -	0
5		Healthy	13 14 15 16 17 18 19 20	- - - - - - - -	0

TABLE NO.6.7T

TEST : 10000 IU VITAMIN A ON DAY 10 p.c. (2 h mating)

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	19	Healthy	1	Broad tail, exomphalos	2
			2	Broad tail	
			3	" "	
			4	" "	
2	19	Healthy		1 fresh IUD, broad tail	
			5	Broad tail	
			6	Small frontal blebs and broad tail	
			7	Broad tail	
			8	" "	
			9	" "	
			10	" "	
3	19	Healthy	11	Broad tail	1
			12	Absent tail, exomphalos	
			13	Broad tail	
			14	" "	
			15	" "	

TABLE NO. 6.7C

CONTROL

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	19	Healthy	1 2 3 4 5 6 7 8	2 fresh, small IUD's (twins)	0
2	19	Healthy	9 10 11 12		1
3	19	Healthy	13 14 15 16		1

TEST GROUP

TABLE NO. 6.8T

10000 IU VITAMIN A ON DAY 10 p.c. (2 hour mating)

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	19	Healthy	1 2 3 4 5 6 7		1
2	19	Healthy	8 9 10 11 12 13 14	1 Macerated IUD Exophthalmos, absent tail " " Slightly prominent eyes " " " " " " " " " " " "	0
3	19	Healthy	15 16 17 18 19	 Nasal blebs, broad tail " " " "	1

TABLE NO. 6.8C

CONTROL GROUP

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	19	Healthy	1 2 3 4 5 6 7 8		0
2	19	Healthy	9 10 11 12 13 14		0
3	19	Healthy	15 16 17 18 19 20 21	Fresh IUD - blood stained liquor	1

TABLE NO. 6.9T

TEST : 5000 IU VITAMIN A ON DAY 9½ p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1 2 3 4 5 6 7	Normal	1
2	18½	Healthy	8 9 10 11 12	Normal	0
3	18½	Healthy	13 14 15 16 17 18 19	? Mild exophthalmos - ? Mild exophthalmos	0

TABLE NO. 6.9T (Contd.) TEST : 5000 IU VITAMIN A ON DAY 9½ p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
4	18½	Healthy	20		0
			21		
			22		
			23		
			24		
			25		
			26		
			27	Exomphalos	
			28		

TABLE NO. 6.9C

CONTROL

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1 2 3 4 5 6		1
2	18½	Healthy	7 8 9) 10) 11	1 macerated IUD Twins, small Twins, small	0
3	18½	Healthy	12 13 14 15 16 17 18) 19)	 Twins, small Twins, small	0
4	18½	Healthy	20-27		0

TABLE NO. 6.1OT

TEST : 1000 IU VITAMIN A ON DAY 9½ p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1 2 3 4 5 6 7		0
2	18½	Healthy	8 9 10 11 12 13 14	1 fresh IUD - no gross abnormalities	0
3	18½	Healthy	15 16 17 18 19		1

TABLE NO. 6.1oc

CONTROL

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	18½	Healthy	1 2 3 4 5 6 7		o
2	18½	Healthy	8 9 10 11 12 13		1
3	18½	Healthy	14 15 16 17 18	1 macerated IUD	o

TABLE NO. 6.12T

TEST : 10 000 IU VITAMIN A ADMINISTERED ON DAY 17 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	-	18	40	Healthy	1	1200		0	78,7	3,10
					2	1280			86,9	3,42
					3	1360			84,6	3,33
					4	1140			79,8	3,30
					5	1370	Exomphalos		87,7	3,43
					6	1290			81,2	3,46
					7	1350			87,4	3,29
2	-	18	41	Healthy	8	1220		1	82,1	2,94
					9	1270			86,4	3,44
					10	1280			83,6	3,07
					11	1340			86,1	3,18
					12	1310			84,0	3,65
					13	1250			85,1	3,36
3	-	18	39,5	Healthy	14	1290		1	83,8	3,32
					15	1340			86,6	3,43
					16	1320			87,2	3,39
					17	1330			85,0	3,43
					18	1190			84,0	3,59
					19	1225			86,5	3,36

TABLE NO. 6.12C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	-	18	38,5	Healthy	1	1300	1 small IUD	1	86,1	3,33
					2	1390			84,2	3,46
					3	1230			83,1	3,18
					4	1250			84,9	3,26
					5	1130			76,9	3,34
2	-	18	40,5	Healthy	6	1050		0	81,2	3,25
					7	1280			84,2	3,41
					8	1270			81,0	3,20
					9	1360			85,4	3,34
					10	1300			83,5	3,03
					11	1260			84,2	3,52
					12	1330			84,3	2,86
3	-	18	39,0	Healthy	13	1280		0	84,2	3,41
					14	1270			81,0	3,20
					15	1360			85,4	3,34
					16	1300			83,5	3,03
					17	1260			84,2	3,27
					18	1330			84,3	3,18

TABLE NO.6.17T

TEST : 1000 IU VITAMIN A ON DAY 9 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	Not done	18	Not done	Healthy			1 IUD - no gross abnormalities	0		
					1	993,2			68,1	2,80
					2	1003,0			69,5	2,81
					3	1021,0			63,2	2,33
					4	926,5			67,3	2,50
					5	935,3			68,9	2,245
					6	979,2			67,5	2,54
					7	913,3			64,7	2,27
2	Not done	18	Not done	Healthy			2 fresh IUD's - no gross abnormalities	0		
					8	994,8			70,7	2,82
					9	984,3			68,1	2,60
					10	939,1			67,0	2,49
					11	933,5			67,0	2,44
3	Not done	18	Not done	Healthy				1		
					12	810,3			60,8	2,11
					13	831,9			61,8	2,29
					14	854,0			61,5	2,08
					15	866,6			63,6	2,24
					16	782,4			59,5	2,29
					17	810,4			59,2	2,38
					18	958,2			67,3	2,02
					19	871,2			63,3	2,11
					20	797,5			64,1	2,07

TABLE NO. 6.17T (Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
4	Not done	18	Not done	Healthy	21	849,7		0	59,4	2,21
					22	747,1			53,6	1,90
					23	773,4			59,7	2,09
					24	817,6			63,6	1,96
					25	624,8			48,0	2,03
					26	744,3			63,3	1,94
					27	840,8			59,9	2,17

TABLE NO. 6.17C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	Not done	18	Not done	Healthy	1	1017,2		1	63,5	2,35
					2	1073,4			72,1	2,86
					3	930,7			67,7	2,53
					4	1042,9			70,9	2,71
					5	976,8			69,8	2,60
2	Not done	18	Not done	Healthy	6	934,3		0	67,2	2,58
					7	961,8			66,8	2,29
					8	1062,7			74,3	2,57
					9	1014,3			69,5	2,26
					10	1021,7			73,3	2,14
					11	978,5			68,6	2,66
					12	794,3			61,5	2,08
3	Not done	18	Not done	Healthy	13	763,8		0	57,9	2,75
					14	751,8			58,4	2,27
					15	802,4			61,2	2,13
					16	702,5			55,9	1,87
					17	797,0			60,0	2,14
					18	803,3			57,1	1,99
					19	835,5			64,2	2,24
					20	698,3			51,4	2,23
					21	587,7			47,1	2,0

TABLE NO. 6.17C (Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
4	Not done	18	Not done	Healthy	22	684,5	1 macerated IUD	0	55,4	2,28
					23	758,4			59,6	2,43
					24	768,2			59,7	2,27
					25	752,9			62,1	1,63
					26	718,8			59,9	2,34
					27	782,7			58,6	2,38
					28	696,1			53,7	2,25
					29	759,6			63,0	2,22
					30	733,9			59,2	2,21

TABLE NO. 6.19T

TEST : 5000 IU VITAMIN A ON DAY 9 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	24,0	18	37,5	Healthy	1	1032,9		1	70,4	2,86
					2	1020,2			71,6	2,71
					3	1059,0			75,1	2,86
					4	941,6			71,5	2,81
					5	990,8			71,6	2,78
					6	706,7	Runt		52,1	2,60
					7	969,8			71,5	2,43
2	27,5	18	42,0	Healthy	8	954,0		2	66,6	2,60
					9	1074,1			71,5	2,76
					10	1039,4			70,9	3,01
					11	826,3	Exophthalmos		62,2	2,66
					12	829,6	"		60,1	2,20
					13	864,5			64,6	2,37
					14	1052,5			70,6	2,90
					15	1010,2			72,8	2,83
3	26,5	18	34,5	Healthy	16	1167,2		2	77,2	2,66
					17	1005,8			73,0	2,70
					18	1100,2			76,9	2,78
4	24,0	18	38,0	Healthy			1 macerated IUD 2 fresh IUD's - no gross abnormalities	0		
					19	857,2			67,7	2,48

TABLE NO.6.19T(Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
4	24,0	18	38,0	Healthy	20	1055,8		0	80,3	3,06
					21	820,0			68,9	2,56
					22	864,0			68,9	2,50

TABLE NO. 6.19C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	25,5	18	37,5	Healthy	1	1045,0		2	70,6	2,70
					2	1009,7			70,1	2,80
					3	901,9			59,2	2,70
					4	969,2			72,0	2,56
					5	1056,8			74,4	2,78
					6	1082,5			74,7	2,71
					7	1038,9	Haemorrhagic blebs over dorsal mid-line of nose		72,0	2,71
2	28	18	41	Healthy	8	925,7		1	66,3	2,63
					9	1086,0			71,3	2,99
					10	954,8			68,5	2,81
					11	1014,6			67,7	2,76
					12	1023,8			70,8	2,76
					13	1023,1			67,7	2,95
					14	1141,3			72,3	2,75
3	25,5	18	34,5	Healthy	15	1119,6		0	74,8	2,85
					16	1072,6			75,0	2,98
					17	925,8			65,0	2,48
					18	824,2	Runt		60,7	2,60
					19	1123,2			77,0	2,86
					20	654,8	Runt		56,8	2,58

TABLE NO. 6.21T

TEST : VITAMIN A 5000 IU ON DAY 10 p.c. (sacrificed on day 14)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Head weight in mg	Brain AChE in nmol/min/mg
1	21,5	14	30,0	Healthy	1	195,6	Normal	0	77,6	0,913
					2	153,0	"		64,0	0,87
					3	152,1	"		55,1	0,913
					4	178,1	"		70,7	0,957
					5	183,8	"		76,0	0,913
					6	195,7	"		69,0	0,957
2	23,5	14	29	Healthy	7	179,1	Normal	2	76,5	0,957
					8	163,9	"		67,7	0,892
					9	179,2	"		73,9	0,892
					10	201,2	"		80,4	0,979
3	22,0	14	31,5	Healthy	11	204,3	Normal	0	68,1	1,12
					12	199,0	"		64,4	1,088
					13	202,0	"		73,5	1,077
					14	205,5	"		71,4	1,153
					15	182,3	"		65,7	1,207
					16	203,0	"		73,1	1,066
					17	149,7	"		53,8	0,957
4	22,0	14	31,5	Healthy	18	176,0	Normal	0	60,2	1,142
					19	193,2	"		68,6	1,153
					20	167,9	"		61,6	0,924
					21	166,0	"		58,1	1,022
					22	176,7	"		65,7	1,196

TABLE NO. 6.21C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Head weight in mg	Brain AChE in nmol/min/mg
1	20	14	31,5	Healthy	1	190,1	Normal	0	76,2	1,066
					2	206,9	"		77,2	0,957
					3	189,0	"		75,1	0,935
					4	198,1	"		73,1	1,077
					5	154,7	"		59,3	0,892
					6	156,5	"		66,4	1,055
2	21,0	14	31,5	Healthy	7	189,4	Normal	0	68,5	1,001
					8	180,6	"		65,3	1,392
					9	195,3	"		72,5	1,001
					10	203,1	"		67,6	0,957
					11	185,0	"		73,0	1,022
					12	179,7	"		68,6	1,001
					13	195,8	"		69,7	1,109
3	19,5	14	30	Healthy	14	172,9	Normal	2	55,0	1,022
					15	174,9	"		62,3	1,066
					16	177,8	"		65,6	0,957
4	20,0	14	31,0	Healthy	17	203,5	Normal	0	72,7	1,109
					18	186,5	"		62,8	1,001
					19	178,6	"		64,2	1,044
					20	186,2	"		60,9	1,066
					21	195,6	"		69,3	1,044
					22	208,5	"		74,7	1,088

TABLE NO. 6.23T

TEST : 5000 IU VITAMIN A ON DAY 10 p.c. (sacrifice day 12)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Head weight in mg	Brain AChE in nmol/min/mg
1	25	12	30	Healthy	1	53,8		0	13,5	0,914
					2	70,8			22,7	0,979
					3	64,9			19,1	0,75
					4	78,4			19,5	0,816
					5	65,7			20,6	0,816
					6	73,2			17,6	0,816
2	25	12	29	Healthy	7	61,9		0	19,8	0,783
					8	72,9			26,5	0,718
					9	74,8			26,1	0,816
					10	82,1			30,3	0,652
					11	74,5			22,8	0,718
					12	60,1			16,3	0,816
					13	74,8			23,4	0,750
					14	68,9			19,1	0,652
3	23,5	12	27	Healthy	15	70,2		0	26,1	0,750
					16	75,7			27,4	0,750
					17	79,1			26,2	0,718
					18	81,2			28,9	0,685
					19	71,6			21,7	0,685
					20	74,2			25,6	0,685

TABLE NO. 6.23C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Head weight in mg	Brain AChE in nmol/min/mg
1	25	12	30	Healthy	1	35,1		0	8,0	0,979
					2	54,8			19,3	0,816
					3	55,8			15,6	0,816
					4	54,7			15,6	0,816
					5	65,9			18,9	0,767
					6	59,4			15,8	0,995
					7	43,1			13,9	0,914
					8	53,7			14,6	0,979
2	23	12	27,5	Healthy	9	70,5		1	20,8	0,75
					10	80,4			26,0	0,734
					11	74,4			27,2	0,75
					12	78,9			27,4	0,75
					13	67,5			18,4	0,783
					14	75,4			18,5	0,848
					15	86,3			27,4	0,718
3	25	12	30	Healthy	16	59,6		1	20,3	0,685
					17	64,5			21,7	0,75
					18	71,7			20,8	0,718
					19	56,9			17,3	0,685
					20	67,0			21,2	0,75

TABLE NO. 6.25T

TEST : VITAMIN A 10000 IU ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	23	18	34,5	Healthy			1 fresh IUD - short tail			
					1	1028,2	Short tail	2	68,6	3,42
					2	1059,4	" "		68,6	3,56
					3	999,3	" "		67,0	3,29
					4	1043,8	" "		76,2	3,36
					5	1072,3	" "		70,5	3,07
2	22,5	18	35,5	Healthy	6	1007,8		1	73,4	3,16
					7	1042,9			74,5	3,36
					8	1028,4			74,6	3,26
					9	1117,8			79,5	3,42
					10	1031,9			77,5	3,26
					11	1041,3			72,0	3,16
					12	1233,7			84,7	3,49
3	21,5	18	34,5	Healthy			1 fresh IUD			
					13	1140,3		0	77,6	3,31
					14	1171,6	Curled tail		77,7	3,07
					15	1156,9	" "		76,6	3,13
					16	985,7	" "		72,3	3,10
					17	1016,0	" "		74,1	3,20
					18	1062,0	" "		76,1	3,07

TABLE NO. 6.25T (Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
4	22	18	35,5	Healthy	19	740,4	1 macerated IUD Exophthalmos, absent tail	0	55,7	2,95
					20	706,2	" "		55,7	2,61
					21	933,2	-		67,1	3,05
					22	926,9	-		69,9	2,89
					23	889,5	-		68,4	2,94
					24	958,6	-		71,5	2,81
					25	876,8	-		68,9	2,74
					26	1064,1	-		77,6	3,16

TABLE NO. 6.25C

CONTROL GROUP

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	23	18	36,5	Healthy	1	1039,5	1 fresh IUD	0	69,5	3,2
					2	976,8			68,0	3,05
					3	1080,4			71,3	2,85
					4	1024,7			68,8	3,03
					5	1101,3			72,6	2,94
					6	966,9			69,9	2,89
					7	1004,9			70,0	2,94
2	21,5	18	32,5	Healthy	8	1098,7	2 macerated IUD's	1	70,3	2,94
					9	1048,9			67,8	3,13
					10	1126,3			70,6	2,89
3	22	18	35	Healthy	11	986,8		1	65,6	2,97
					12	920,2			64,0	2,90
					13	989,4			67,9	2,81
					14	889,4			67,0	2,89
					15	952,3			64,6	2,77
					16	1016,9			69,2	2,84

TABLE NO.6.25c (Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
4	23	18	37	Healthy	17	1039,4		0	70,1	3,10
					18	958,4			64,6	3,23
					19	902,9			63,5	3,26
					20	929,6			66,2	3,10
					21	943,1			65,5	3,00
					22	1049,7			68,9	3,13
					23	1030,5			68,5	2,97
					24	1048,6			70,5	3,07

TABLE NO. 6.26T

TEST : 10000 IU VITAMIN A ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	25	19	41	Healthy	1	1283,4	Normal	0	87,0	3,59
					2	1254,1			85,4	3,64
					3	1357,9			88,2	3,47
					4	1219,6			82,0	3,51
					5	1225,4			83,1	3,40
					6	1267,7			85,2	3,56
					7	1308,9			88,7	3,39
2	25	19	35,5	Healthy	8	1306,6	Short forelimbs, broad tail	2	93,4	3,26
					9	1349,8	" "		93,8	3,65
					10	1205,4	" "		86,7	3,29
3	26	19	41,5	Healthy	11	1175,7	2 macerated IUD's with broad tails Short forelimbs, broad tail, nasal blebs †	0	84,9	3,54
					12	1029,2	" " "		80,6	3,38
					13	1106,3	" " "		83,3	3,65
					14	1071,5	" " "		78,3	3,69
					15	1029,6	" " "		80,8	2,94
					16	1246,5	" " "		90,3	2,91
					17	1081,5	" " "		85,7	3,52

† Nasal blebs : ± 1mm cystic blebs, one on either side of midline over maxillo-frontal region

TABLE NO.6.26T(Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
4	25	19	37	Healthy	18	1357,3	-	1	91,4	3,82
					19	1288,3	Nasal blebs, broad tail		89,1	3,69
					20	1213,7	Nasal blebs, broad tail, hydramnios		89,7	3,78
					21	1228,2			88,1	3,75
					22	1225,6			87,1	3,55

TABLE NO. 6.26C

CONTROL GROUP

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	27	19	40	Healthy	1	1455,5		1	90,8	3,59
					2	1249,8			87,1	3,39
					3	1374,2			83,8	3,10
					4	1292,1			85,5	3,29
					5	1300,1			86,8	3,59
					6	1382,1			88,2	3,41
2	27	19	39,5	Healthy	7	1309,3		1	82,9	3,10
					8	1246,5			82,5	3,00
					9	1356,9			86,1	3,28
					10	1120,6			78,6	3,33
					11	1266,5			85,7	3,14
					12	830,4			66,4	2,74
3	25	19	34,5	Healthy	13	1054,8		0	73,6	2,64
					14	929,8			73,3	2,69
					15	1286,3			87,6	2,94
					16	1140,9			78,8	2,95
4	24	19	35	Healthy	17	1031,3		0	74,1	3,28
					18	1252,7			85,6	3,36
					19	1353,5			99,6	2,98
					20	1350,9			90,4	3,40
					21	1370,9			92,7	3,39

TABLE NO. 6.29T

TEST : 10000 IU VITAMIN A ON DAY 13 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	26	18	31,5	Healthy	1	1050,0		0	72,9	2,51
					2	1020,0			62,8	2,15
					3	1100,0			76,5	2,53
					4	880,0			68,0	2,25
					5	1030,0			72,3	2,46
					6	950,0			70,7	2,32
2	30	18	35,5	Healthy	7	730,0	1 macerated IUD	0	62,0	1,99
					8	960,0			71,0	2,69
					9	880,0			73,2	2,28
					10	860,0			70,8	2,28
					11	940,0			71,7	2,40
					12	950,0			75,6	2,58
					13	1030,0			75,9	2,64
3	28	18	32,5	Healthy	14	1110,0	1 fresh IUD	0	81,3	2,87
					15	860,0			77,7	2,81
					16	1040,0			77,7	2,56
					17	1070,0			83,2	2,64
					18	1030,0			78,8	2,68
					19	1020,0			79,6	2,50

TABLE NO. 6.29c

CONTROL GROUP

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	22	18	29	Healthy	1	890,0		0	67,4	2,38
					2	970,0			69,0	2,53
					3	850,0			64,5	2,25
					4	820,0			63,0	2,22
					5	850,0			62,3	2,38
					6	920,0			66,5	2,30
					7	760,0			62,7	2,32
2	30	18	35,5	Healthy	8	910,0		0	71,3	2,38
					9	1000,0			69,4	2,69
					10	1000,0			71,8	2,59
					11	1020,0			74,3	2,38
					12	940,0			74,2	2,53
					13	900,0			70,3	2,28
					14	1020,0			72,6	2,27
3	31	18	38,5	Healthy			1 fresh IUD - no gross abnormalities			
					15	1120,0			77,8	2,56
					16	1050,0			77,7	2,68
					17	1050,0			77,2	2,63
					18	1160,0			79,1	2,61
					19	1050,0			74,6	2,50
					20	950,0			72,1	2,27
					21	1180,0			80,2	2,56

TABLE NO. 6.31T

TEST : VITAMIN A 10000 IU ON DAY 9 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	27,5	19	41,5	Healthy	1	1340	Kinky tail	1	92,8	3,65
					2	1200	" "		84,8	3,13
					3	1280	" "		92,3	3,62
					4	1240	Broad tail		86,1	3,36
					5	1070	Exophthalmos, kinky tail		84,4	3,42
					6	1170	" "		75,3	3,28
2	24,5	19	40	Healthy	7	1190	1 fresh IUD with exophthalmos Exophthalmos, absent tail		75,4	3,52
					8	960	" "		71,0	3,20
					9	1070	" "		70,7	3,20
					10	1080	" "		72,5	3,23
					11	1200	Prominent eyes, absent tail		79,8	3,62
					12	920	" "		72,9	3,31
					13	1000	Exophthalmos, absent tail, exomphalos		68,8	3,23

TABLE NO. 6.31T (Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
3	24,5	19	39	Healthy			1 fresh IUD with exophthalmos and absent tail			
					14	1240	Exophthalmos, absent tail	1	83,4	3,36
					15	1150	" "		77,4	3,46
					16	1250	" "		82,8	3,51
					17	1200	" "		79,3	3,20
					18	1250	Exophthalmos, absent tail, open spina bifida, hydrammios		76,1	3,60
4	26,5	19	36,5	Healthy	19	1300	Exophthalmos, kinky tail	2	86,6	3,36
					20	1050	" "		70,2	3,47
					21	1250	Exophthalmos, kinky tail, open spina bifida, blood stained hydrammios		70,2	3.29

TABLE NO. 6.31C

CONTROL GROUP

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	22	19	38	Healthy	1	1000	Normal	0	76,2	3,56
					2	1220	"		83,4	3,47
					3	1390	"		85,2	3,36
					4	1250	"		84,5	3,36
					5	1190	"		80,6	3,15
					6	1190	"		86,7	3,42
					7	1250	"		82,1	3,46
2	23	19	34	Healthy	8	1460	Normal	0	86,9	3,36
					9	1400	"		85,0	3,23
					10	1470	"		89,1	3,33
					11	1330	"		84,0	3,47
3	25,5	19	43	Healthy	12	1250	Normal	0	82,8	3,36
					13	1320	"		86,2	3,39
					14	1170	"		79,2	3,18
					15	1210	"		82,4	3,60
					16	1040	"		70,4	3,33
					17	1230	"		80,5	3,44
					18	1250	"		79,7	3,49
					19	1170	"		78,8	2,89

TABLE NO. 6.33T

TEST : VITAMIN A 10 000 IU ON DAY 9 p.c. (SACRIFICE DAY 12 p.c.)

FIRST EXPERIMENT

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	24,0	12	28,5	Healthy	1	78,2		0	39,7	Spoilt
					2	87,8			47,8	Spoilt
					3	84,5			40,0	Spoilt
					4	69,6			36,3	0,9462
					5	71,1			34,2	0,5873
					6	75,1			38,7	0,6199
					7	64,0			31,0	0,7831
					8	69,5			34,8	0,5221
2	22,5	12	25,5	Healthy	9	51,0		0	22,4	0,7831
					10	80,0			32,2	0,7831
					11	77,1			30,6	0,6852
					12	63,0			30,4	0,6526
					13	75,0			25,6	0,7505
					14	76,7			33,3	0,5873
					15	61,6			25,8	0,6852
					16	76,8			28,4	0,6852
3	23,0	12	26,5	Healthy	17	80,8		0	28,4	0,6199
					18	79,9			30,0	0,6852
					19	87,7			29,8	0,7178
					20	90,3			33,0	0,6852
					21	79,2			29,2	0,6199
					22	68,2			29,4	0,4894
					23	74,4			29,2	0,7178
					24	78,9			30,4	0,5873

TABLE NO. 6.33C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	23,0	12	27	Healthy	1	71,6		1	42,7	Spoilt
					2	82,1			48,1	Spoilt
					3	68,9			42,0	Spoilt
					4	67,5			36,6	0,5547
					5	67,9			40,9	0,9462
					6	65,3			31,0	0,7505
					7	58,0			32,4	0,4568
					8	57,5			28,8	0,6199
2	24,0	12	27	Healthy	9	66,0		0	24,6	0,8157
					10	67,0			21,2	1,0115
					11	78,5			25,6	0,9136
					12	86,3			26,1	0,8810
					13	62,1			20,7	0,6199
					14	80,4			24,6	0,5547
					15	84,1			31,5	0,6526
					16	66,1			24,6	0,7831
3	23,5	12	27,5	Healthy	17	84,5		0	31,3	0,6199
					18	67,1			23,5	0,6852
					19	90,3			32,1	0,6526
					20	80,1			32,3	0,6526
					21	81,6			25,9	0,8810
					22	79,5			26,3	0,7505
					23	63,4			24,2	0,7831

TABLE NO. 6.35T TEST : VITAMIN A 10 000 IU ON DAY 9 p.c. (SACRIFICE DAY 12 p.c.)
SECOND EXPERIMENT

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	24,0	12	27,5	Healthy	1	70,8	Haemorrhagic areas in brain	0	26,6	0,6342
					2	74,4			30,9	0,7188
					3	71,1			27,9	1,0570
					4	59,7			18,1	0,8033
					5	72,0			27,6	0,7188
					6	73,2			27,6	0,7610
					7	63,1			23,5	0,5074
					8	71,2			17,9	0,6765
2	23,5	12	27,0	Healthy			3 IUD's, haemorrhagic necrotic fetal tissue, blood stained amniotic fluid			
					9	73,2		0	27,5	0,9301
					10	68,7			22,7	0,7610
					11	60,1	Small haemorrhagic areas in brain		17,9	0,8879
					12	75,6			23,3	0,5496

TABLE NO. 6.35T

TEST : VITAMIN A 10 000 IU ON DAY 9 p.c. (SACRIFICE DAY 12 p.c.)

SECOND EXPERIMENT

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
3	23,0	12	27,0	Healthy	13	78,8	Small haemorrhagic areas in brain	0	21,3	0,4651
					14	58,0			18,1	0,9301
					15	70,7			17,9	0,9301
					16	69,1			29,9	0,5496
					17	74,0			22,2	0,8033
					18	62,3			17,5	0,7610
					19	70,7			24,6	0,8033
					20	69,1	Hypoplastic face		28,8	0,8033

TABLE NO. 6.53T

VITAMIN A 15 000 IU ADMINISTERED ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain ChAT activity as d.p.m *
1	23,0	18	26,5	Healthy			(7 late resorptions - ± 4mm necrotic fetal remnants with well developed placentae)	7		
2	22,5	18	36,0	Healthy	1	0,905	Nasal blebs, kinked tails, spade-like limbs	0	70,4	50
					2	0,943	" "		62,6	344
					3	0,980	" "		74,3	113
					4	0,933	" "		70,8	76
					5	0,965	" "		72,5	570
					6	0,907	" "		57,3	425
					7	0,957	" "		71,0	462
3	23,5	18	30,5	Healthy	8	0,942	Exomphalos, kinked tail	3	69,1	394
					9	1,084	Kinked tail		73,1	226
					10	1,088	Kinked tail		74,8	576
4	22,5	18	27,5	Healthy			(6 late resorptions - ± 4mm necrotic fetal remnants, placentae well developed)	6		

* Actual d.p.m. (blank value subtracted)

TABLE NO. 6.53T(Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain ChAT activity as d.p.m.*
5	23,0	18	33	Healthy			1 ± 17 day IUD nasal blebs, kinked tail. (1 late resorption ± 4mm necrotic fetal remnants well developed placentae	1		
					11	0,977	Nasal blebs, kinked tail, spade like limbs		72,3	485
					12	0,937	" " "		67,6	467
					13	0,863	" " "		62,8	584
					14	0,919	" " "		66,9	406
					15	0,919	" " "		66,3	181
6	23,5	18	29	Healthy			6 late resorptions as in mouse 1 above	6		

* Actual d.p.m. (blank value subtracted)

TABLE NO. 6.53C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain ChAT activity as d.p.m *
1	23	18	35	Healthy	1	1,083		0	73,3	171
					2	0,940			66,9	424
					3	0,928			69,8	41
					4	0,970			65,6	254
					5	0,961			71,6	525
					6	1,048			65,6	448
2	22,5	18	37	Healthy	7	0,989	2 IUD's ± 17 days - no abnormalities	0	63,7	502
					8	1,001			66,8	59
					9	1,132			71,4	819
					10	1,087			69,1	665
					11	1,004			67,1	563
					12	0,877			66,0	424
					13	1,119			65,9	127
3	23,5	18	37	Healthy	14	0,937		0	66,8	371
					15	0,966			70,2	443
					16	0,974			73,3	271
					17	0,968			71,1	564
					18	1,005			68,8	609
					19	1,002			69,2	291
					20	1,068			55,6	435
					21	1,017			69,1	682

* Actual d.p.m. (blank value subtracted)

TABLE 6.55T

METHOD 1 : ANALYSIS OF ISOENZYMES. PEAK HEIGHTS (mm) OF PEAKS 1-5 AND THE SUM OF PEAKS 1-5 ("TOTAL")

Sample No.	TEST Peak heights (mm)					
	1	2	3	4	5	TOTAL
1	69,5	121,0	102,0	55,0	79,5	427,0
2	53,0	108,0	89,0	42,5	85,0	377,5
3	62,0	114,5	109,0	60,5	91,5	437,5
4	73,5	126,0	91,0	61,5	65,0	417,0
5	50,5	108,5	83,5	47,5	82,5	378,5
6	38,5	87,0	59,0	22,0	99,0	305,5
7	43,5	79,0	56,0	34,0	85,5	298,0
8	53,0	92,5	72,0	49,0	70,0	336,5
9	50,5	87,0	68,0	34,0	78,0	317,5
10	32,0	73,5	58,5	26,5	69,0	259,5
11	48,5	102,0	90,0	59,0	88,0	387,5
12	43,5	102,5	99,5	56,5	103,0	405,0
13	63,5	123,0	105,5	48,0	112,5	452,5
14	70,5	124,5	112,5	63,0	98,5	469,0
15	45,0	71,0	64,5	47,5	56,5	284,5
16	52,0	86,0	80,0	61,0	69,5	348,5
17	49,5	89,5	79,0	68,5	77,0	363,5
18	43,5	82,5	76,0	40,5	52,5	295,0
19	56,0	96,5	97,5	69,5	58,0	377,5
20	57,5	98,0	95,0	65,0	67,0	382,5
Median	52,75	98,13	84,5	51,5	78,6	366,8
95% Confidence limits	47,75 to 58,5	89,5 to 106,8	75,5 to 94,0	44,00 to 58,5	70,75 to 87,75	336,5 to 397,3
p *	N.S.	N.S.	N.S.	N.S.	0,05	N.S.

* Comparison with data in Table 6.55C.
For derivation of p value see text.

TABLE 6.55C

METHOD 1 : ANALYSIS OF ISOENZYMES. PEAK HEIGHTS (mm) OF PEAKS 1-5 AND THE SUM OF PEAKS 1-5 ("TOTAL")

Sample No.	CONTROL Peak heights (mm)					
	1	2	3	4	5	TOTAL
1	70,5	114,0	106,0	66,0	45,0	401,5
2	61,0	103,5	100,0	55,5	38,5	358,5
3	57,0	115,5	99,5	51,5	89,0	412,5
4	57,5	101,0	84,0	45,5	68,0	356,0
5	61,5	99,5	75,5	52,0	43,0	331,5
6	57,5	96,5	84,0	47,5	41,0	326,5
7	35,0	76,0	68,5	30,0	78,0	287,5
8	35,5	72,5	59,0	40,5	88,0	295,5
9	40,0	82,0	76,5	32,5	80,5	311,5
10	41,0	72,5	54,4	28,0	70,5	266,5
11	68,0	112,5	108,5	77,0	90,5	456,5
12	50,5	97,0	89,0	43,0	62,0	341,5
13	39,5	89,5	81,0	56,0	89,0	355,0
14	53,0	106,0	98,0	50,5	59,0	366,5
15	62,0	116,0	108,5	65,0	61,5	413,0
16	56,5	107,0	103,0	65,5	45,5	377,5
17	51,0	81,5	79,0	49,0	48,0	308,5
18	28,5	57,0	55,5	36,5	46,0	223,5
19	55,5	100,5	95,5	69,5	72,5	393,5
20	50,0	99,5	92,0	69,5	77,0	388,0
Median	51,88	96,5	87,0	51,13	64,88	350,0
95% Confidence limits	45,75 to 57,5	86,5 to 104,0	77,5 to 95,00	44,25 to 58,75	55,00 to 74,75	321,5 to 377,3

TABLE 6.57T

METHOD 2 : COMPARISON OF TEST AND CONTROL PEAK HEIGHTS USING PEAK THREE AS AN INTERNAL STANDARD

TEST

	PEAK NUMBER				
Sample No.	1	2	4	5	TOTAL
1	0,68	1,19	0,54	0,78	3,19
2	0,6	1,21	0,48	0,95	3,24
3	0,57	1,05	0,56	0,84	3,02
4	0,81	1,38	0,68	0,71	3,58
5	0,68	1,3	0,57	0,99	3,54
6	0,65	1,47	0,37	1,68	4,17
7	0,78	1,41	0,61	1,53	4,33
8	0,74	1,28	0,68	0,97	3,67
9	0,74	1,28	0,5	1,15	3,67
10	0,55	1,26	0,45	1,18	3,44
11	0,54	1,13	0,66	0,98	3,31
12	0,44	1,03	0,57	1,04	3,08
13	0,6	1,17	0,45	1,07	3,29
14	0,63	1,11	0,56	0,88	3,18
15	0,7	1,1	0,74	0,88	3,42
16	0,65	1,08	0,76	0,87	3,36
17	0,63	1,13	0,87	0,97	3,6
18	0,57	1,08	0,53	0,69	2,87
19	0,57	0,99	0,71	0,59	2,86
20	0,61	1,03	0,68	0,71	3,03
Median ratio	0,637	1,184	0,595	0,935	3,358
LL	0,59	1,105	0,535	0,84	3,2
UL	0,68	1,250	0,66	1,065	3,55
P *	N.S.	0,099	N.S.	0,032	0,0124

* Comparison with data in Table 6.57C.
For derivation of p value see text.

TABLE 6.57C

METHOD 2 : COMPARISON OF TEST AND CONTROL PEAK HEIGHTS USING PEAK THREE AS AN INTERNAL STANDARD

CONTROL

	PEAK NUMBER				
Sample No.	1	2	4	5	TOTAL
1	0,67	1,08	0,62	0,42	2,79
2	0,61	1,04	0,56	0,39	2,6
3	0,57	1,16	0,52	0,89	3,14
4	0,68	1,20	0,54	0,81	3,23
5	0,81	1,32	0,69	0,57	3,39
6	0,68	1,15	0,57	0,49	2,89
7	0,51	1,11	0,44	1,14	3,2
8	0,60	1,23	0,69	1,49	4,01
9	0,52	1,07	0,42	1,05	3,06
10	0,75	1,33	0,51	1,29	3,88
11	0,63	1,05	0,71	0,83	3,22
12	0,57	1,09	0,48	0,7	2,84
13	0,49	1,1	0,69	1,10	3,38
14	0,54	1,08	0,52	0,6	2,74
15	0,57	1,07	0,6	0,57	2,81
16	0,55	1,04	0,64	0,44	2,67
17	0,65	1,03	0,62	0,61	2,91
18	0,51	1,03	0,66	0,83	3,03
19	0,58	1,05	0,73	0,76	3,12
20	0,54	1,08	0,76	0,84	3,22
Median ratio	0,595	1,095	0,6	0,77	3,063
LL	0,555	1,065	0,55	0,625	2,92
UL	0,64	1,155	0,65	0,94	3,255

T A B L E 6.58T

METHOD 3 : COMPARISON OF TEST AND CONTROL PEAK HEIGHTS BY DIVIDING ALL DATA BY MEAN FOR CONTROLS FOR EACH GEL

	TEST					
	1	2	3	4	5	TOTAL
1	0,95	1,66	1,40	0,75	1,09	5,85
2	0,73	1,48	1,22	0,58	1,17	5,18
3	0,85	1,57	1,49	0,83	1,26	6,01
4	1,01	1,73	1,25	0,84	0,89	5,65
5	0,97	1,86	1,44	0,82	1,42	6,51
6	0,66	1,50	1,02	0,38	1,71	5,27
7	0,75	1,36	0,96	0,59	1,47	5,13
8	0,91	1,59	1,24	0,84	1,21	5,79
9	0,87	1,50	1,17	0,59	1,34	5,47
10	0,55	1,27	1,01	0,46	1,19	4,48
11	0,63	1,32	1,17	0,77	1,14	5,03
12	0,56	1,33	1,29	0,73	1,34	5,25
13	0,82	1,60	1,37	0,62	1,46	5,87
14	0,92	1,62	1,46	0,82	1,28	6,10
15	0,69	1,08	0,98	0,72	0,86	4,33
16	0,79	1,31	1,21	0,93	1,06	5,30
17	0,75	1,36	1,20	1,04	1,17	5,52
18	0,66	1,26	1,16	0,62	0,80	4,50
19	0,85	1,47	1,48	1,06	0,88	5,74
20	0,88	1,49	1,45	0,99	1,02	5,83
Median Ratio	0,79	1,47	1,243	0,75	1,18	5,483
-	0,72	1,38	1,17	0,66	1,07	5,175
+	0,86	1,56	1,34	0,83	1,3	5,685
p *	NS	0,077	NS	NS	0,0188	0,041

* Comparison with data in Table 6.58C.

For derivation of p value see text.

TABLE 6.58C

METHOD 3 : COMPARISON OF TEST AND CONTROL PEAK HEIGHTS BY DIVIDING ALL DATA BY MEAN FOR CONTROLS FOR EACH GEL

	CONTROL					
	1	2	3	4	5	TOTAL
1	0,97	1,56	1,45	0,91	0,62	5,51
2	0,84	1,42	1,37	0,76	0,53	4,92
3	0,78	1,58	1,36	0,71	1,22	5,66
4	0,79	1,38	1,15	0,62	0,93	3,88
5	0,84	1,36	1,03	0,71	0,59	4,55
6	0,79	1,32	1,15	0,65	0,56	4,47
7	0,61	1,31	1,18	0,52	1,34	4,96
8	0,61	1,25	1,02	0,70	1,52	5,10
9	0,69	1,41	1,32	0,56	1,39	5,37
10	0,71	1,25	0,94	0,48	1,21	4,59
11	0,88	1,46	1,41	1,00	1,18	5,93
12	0,66	1,26	1,16	0,56	0,81	4,45
13	0,51	1,16	1,05	0,73	1,16	4,61
14	0,69	1,38	1,27	0,66	0,77	4,77
15	0,81	1,51	1,41	0,84	0,80	5,37
16	0,73	1,39	1,34	0,85	0,59	4,90
17	0,78	1,24	1,20	0,75	0,73	4,70
18	0,43	0,87	0,85	0,56	0,70	3,41
19	0,85	1,53	1,45	1,06	1,10	5,99
20	0,76	1,51	1,40	1,06	1,17	5,90
Median ratio	0,745	1,375	1,235	0,7225	0,95	4,99
-	0,68	1,3	1,145	0,635	0,765	4,7
+	0,8	1,44	1,32	0,81	1,11	5,3

TABLE NO. 7.2T

TEST : CYCLOPHOSPHAMIDE 12,5mg/kg ON DAY 17 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	-	18	40	Healthy	1	1087,8		1	73,0	2,7626
					2	1144,4			75,7	2,6973
					3	1098,4			75,3	2,7626
					4	1063,9			72,7	2,3493
					5	1164,6			75,8	2,3275
					6	1194,4			77,7	2,6973
2	-	18	37,5	Healthy	7	1057,9			59,0	1,7184
					8	-	Spoilt	-	-	-
					9	1048,1			70,3	2,2840
					10	709,8			52,1	1,8055
					11	972,7			50,8	2,0447
					12	1065,6			59,3	2,0665
3	-	18	39,5	Healthy	13	1260,3			82,0	2,5885
					14	1144,4			77,6	2,2840
					15	1114,6			76,9	2,3058
					16	1054,6			76,0	2,3710
					17	989,5			73,0	2,3275
					18	1036,7			72,7	2,2187
					19	992,6			73,0	2,3492

TABLE NO. 7.2C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	-	18	39,5	Healthy			1 small IUD			
					1	1074,2		0	75,4	2,0230
					2	1056,8			73,9	2,4580
					3	1113,3			74,7	2,6320
					4	1119,9			73,6	2,8496
					5	1024,1			76,9	2,9366
					6	903,9			68,5	2,4580
					7	1061,5			72,0	2,8061
2	-	18	34,5	Healthy	8	1026,4		2	63,0	1,9142
					9	1070,5	Exomphalos		77,2	1,5879
					10	1145,4			67,9	2,3058
					11	1040,2			70,4	2,3275
3	-	18	43	Healthy			1 small IUD			
					12	1054,4			77,2	2,5233
					13	1094,4			80,1	2,4580
					14	1136,5			76,5	2,5450
					15	1134,3			77,9	2,5233
					16	1017,8			75,6	2,2840
					17	903,4			70,3	2,4580
					18	999,6			76,0	2,4363

TABLE NO. 7.5T

TEST : CYCLOPHOSPHAMIDE 20 mg/Kg ON DAY 10 P.C.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	27	19	33	Healthy	-			7		
2	25	19	30,5	Healthy	-			8		
3	25	19	38,5	Healthy	1	888,0	1 macerated IUD	1	63,9	2,81
					2	881,0	Mild exophthalmos Prominent eyes. Head shortnened anteroposteriorly, hypoplastic mandibles		62,0	3,07
					3	859,0	" " "		62,9	2,94
					4	914,0	" " "		65,5	2,81
					5	875,0	" " "		61,7	2,97
4	26	19	31,5	Healthy	-			6		
5	26,5	19	29	Healthy	-			6		
6	25	19	29	Healthy	-			8		
7	27	19	35	Healthy	6	1012	Prominent eyes	3	64,6	3,16
					7	898,0	" "		62,3	2,87
					8	888,0	" "		59,1	3,12
					9	997,0	" "		65,1	2,9
8	25	19	30,5	Healthy	10	838,0	Prominent eyes	4	59,6	2,97
					11	813,0	" "		59,5	3,16

TABLE NO. 7.5C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	24	19	40	Healthy	1	1400	1 macerated IUD	0	92,8	3,49
					2	1363			93,2	3,46
					3	1387			88,6	3,52
					4	1310			87,0	3,47
					5	1314			83,0	3,26
					6	1243			81,6	3,13
2	23,5	19	41	Healthy	7	1361		0	85,5	3,13
					8	992,0			76,2	2,87
					9	1235			78,6	3,07
					10	1337			86,3	3,33
					11	1182			78,9	2,94
					12	1046			72,3	2,97
					13	1074			75,5	3,05
					14	1197			87,5	3,44
3	24,5	19	39	Healthy	15	1325		0	82,9	3,49
					16	1328			84,4	3,49
					17	1256			87,2	3,41
					18	1435			89,9	3,42
					19	1402			88,1	3,36
					20	1328			88,5	3,42

TABLE NO. 7.7T

TEST : CYCLOPHOSPHAMIDE 12,5 mg/kg ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	26,5	19	36	Healthy	1	990,0		2	66,3	2,28
					2	974,0			66,4	2,71
					3	921,0	Mild exophthalmos		66,4	2,38
					4	920,0	" "		65,8	2,71
2	27	19	43	Healthy	5	864,0	Exophthalmos	1	61,5	2,94
					6	943,0	"		64,8	2,87
					7	965,0	"		65,5	2,32
					8	1130,0	Normal		70,0	3,03
					9	1120,0	"		72,1	3,00
					10	911,0	"		66,4	2,70
					11	1093,0	"		67,7	2,87
					12	843,0	"		59,0	1,61
3	27	19	39	Healthy	13	1040,0	3 macerated IUD's Normal	1	74,9	2,84
					14	1090,0	"		72,4	2,67
					15	986,0	"		70,2	3,03
4	25	19	34	Healthy	16	929,0	Normal	3	67,5	2,97
					17	980,0	"		67,1	3,26
					18	895,0	"		69,8	2,94
					19	941,0	"		61,6	3,23
5	25,5	19	37	Healthy	20	1066,0	1 died	0	75,2	2,77
					21	1020,0			70,8	2,67
					22	1167,0			80,1	2,54

TABLE NO. 7.7C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	25	19	34,5	Healthy	1	1291,0	Normal	0	84,0	3,02
					2	1224,0	"		81,3	3,07
					3	1330,0	"		82,4	3,00
					4	1048,0	"		74,2	3,10
2	22,5	19	27 delivered	Healthy	5	1326,0	Normal	0	84,6	3,16
					6	1250,0	"		81,9	3,36
					7	1380,0	"		88,3	3,10
					8	1290,0	"		85,6	3,02
					9	1206,0	"		77,7	2,87
					10	1218,0	"		81,3	3,02
3	25	19	Delivered	Healthy	11		Normal	0	87,4	3,33
					12		"		86,8	3,34
					13		"		85,0	3,28
					14		"		84,1	3,21
					15		"		86,5	3,20
					16		"		88,4	3,49

TABLE NO. 7.9T

TEST : CYCLOPHOSPHAMIDE 12,5 mg/Kg ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	24,5	19	39,0	Healthy	1	1096,4	Normal	0	73,0	3,132
					2	1160,1	"		72,3	2,871
					3	1071,6	"		70,0	2,79
					4	962,0	"		74,6	3,132
					5	1010,4	"		66,1	2,822
					6	1112,3	"		67,6	2,806
					7	958,9	Mild exophthalmos		69,2	2,985
2	24,0	19	35,0	Healthy			1 macerated IUD			
					8	1294,2	Normal	0	81,0	3,10
					9	1166,3	"		70,2	2,708
					10	1141,8	"		76,6	2,855
					11	1173,9	"		77,4	2,806
3	24,0	19	37,5	Healthy			1 macerated IUD			
					12	1157,7	Normal	0	78,9	3,067
					13	1169,9	"		80,7	3,132
					14	1122,1	"		75,5	2,871
					15	1085,6	"		76,7	2,904
					16	1140,4	"		75,1	3,018
					17	1032,4	"		77,0	2,871
					18	1037,5	"		74,2	2,773

TABLE NO. 7.9C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	25,5	19	38	Healthy	1	1389,2	Normal	1	88,4	3,23
					2	1409,0	"		89,6	3,263
					3	1421,9	"		87,1	3,475
					4	1245,0	"		88,9	3,197
							1 fresh IUD			
2	24	19	36	Healthy	5	1358,6	Normal	0	90,1	3,377
					6	1098,6	"		86,6	3,263
					7	1292,8	"		86,8	3,556
					8	1249,9	"		81,6	3,312
3	23,5	19	34	Healthy	9	1281,4	Normal	1	88,4	3,214
					10	1402,4	"		87,7	3,426
					11	1371,5	"		85,0	3,067
					12	1165,0	"		74,3	3,491
4	24	19	34	Healthy	13	1340,1	Normal	1	86,4	3,393
					14	1158,8	"		75,0	3,295
					15	1207,3	"		81,0	3,10
					16	1288,1	"		87,2	2,936
					17	1417,8	Exomphalos		80,6	2,822
					18	1569,9			102,8	3,328
					19	1359,0			90,6	3,132

TABLE NO. 7.11T

TEST : CYCLOPHOSPHAMIDE 12,5 mg/Kg ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	27,0	18	40,0	Healthy			2 IUD's (near term). No gross abnormalities	0		
					1	814,1	Normal (Small)		57,4	2,708
					2	812,4	" "		53,5	2,708
					3	915,0	" "		62,6	2,969
					4	880,8	" "		60,8	2,806
					5	756,3	" "		57,0	2,643
2	23,5	18	37,5	Healthy			1 IUD ± 14 days			
					6	739,8	Normal (Small)	0	52,5	2,317
					7	803,1	" "		54,6	2,545
					8	729,8	Exophthalmos		55,3	2,447
					9	749,8	Normal		57,0	2,610
					10	755,6	"		54,9	2,578
					11	822,1	"		55,9	2,545
					12	727,5	"		54,6	2,708
3	24,0	18	37,5	Healthy			1 IUD ± 14 days			
					13	916,2	Normal	1	58,3	2,741
					14	823,5	"		55,9	2,317
					15	818,7	"		57,9	2,382
					16	865,8	"		58,4	2,545
					17	937,9	"		61,4	3,035

TABLE NO. 7.11T (Contd)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
4	26,5	18	38	Healthy	18	837,0	Normal	1	54,3	2,088
					19	985,7	"		64,7	2,806
					20	937,8	"		61,6	2,480
					21	983,5	"		62,6	2,643
					22	848,9	"		60,0	2,839

TABLE NO. 7.11C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	22,5	18	34,5	Healthy			1 IUD			
					1	1105,5	Normal	0	74,0	3,035
					2	991,1	"		71,1	3,035
					3	1032,7	"		70,6	3,10
					4	997,9	"		68,7	2,904
					5	1013,0	"		69,0	3,002
2	21,5	18	35,0	Healthy	6	1082,7	Normal	1	71,9	2,904
					7	1103,3	"		73,5	3,035
					8	1068,3	"		71,9	2,937
					9	1053,4	"		70,7	3,002
					10	1055,5	"		69,1	2,839
3	27,5	18	46	Healthy			1 Fresh IUD			
					11	1049,4	Normal	0	72,2	2,937
					12	974,6	"		63,6	2,480
					13	954,1	"		67,4	2,806
					14	989,3	"		71,0	2,708
					15	1010,1	"		69,6	2,806
					16	949,8	"		67,9	2,871
					17	990,6	"		66,2	2,969
					18	1090,9	"		74,6	3,035
					19	1009,7	"		69,7	3,035

TABLE NO. 7.14T TEST : CYCLOPHOSPHAMIDE 15 mg/kg ON DAY 10 p.c. (SACRIFICE DAY 18 p.c.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	25	18	36	Healthy	1	761,0		0	56,1	2,3058
					2	831,1			55,7	2,5015
					3	796,1			56,0	2,6538
					4	749,0			57,0	2,4580
					5	771,0			54,7	2,3058
					6	792,6			54,8	2,6103
					7	769,2			57,9	2,2623
2	24	18	32	Healthy	-	-	3 I.U.D.'s (± day 12 - 14 fetuses with hydramnios)	6		
3	25	18	34	Healthy			3 I.U.D.'s (± day 14 fetuses with hydramnios)	0		
					8	777,9			57,4	2,2623
					9	713,5			58,7	2,1535
					10	823,6			56,9	2,5450
					11	820,9			60,5	2,2405
4	24	18	28	Healthy	-	-	1 I.U.D. (± 14 day fetus with hydramnios)	4		
5	26	18	32	Healthy	-	-	2 I.U.D.'s (± 12 day fetuses)	5		

TABLE NO. 7.14T(Contd.)

TEST

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
6	22	18	32	Healthy	12	913,0		0	62,9	2,5015
					13	962,0			64,3	2,6103
					14	790,9			60,0	2,1100
					15	840,3			61,3	2,4580
					16	826,0			60,2	2,2840
7	24	18	31	Healthy	17	669,7	Exophthalmos	5	51,0	2,3710

TABLE NO. 7.14C

CONTROL : (SACRIFICE DAY 17 p.c.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	24	17	35	Healthy	1	732,0		0	56,6	1,6967
					2	716,5			57,3	2,0665
					3	578,8			48,6	1,8707
					4	727,3			56,5	2,0012
					5	704,4			46,7	1,6314
					6	772,5			53,4	1,8272
					7	735,0			55,4	1,9360
2	24	17	35	Healthy			1 Fresh I.U.D. - no abnormalities			
					8	676,8	-	0	53,7	1,9577
					9	726,4			57,1	2,0447
					10	624,0			56,2	1,8055
					11	667,3			54,9	2,0230
					12	795,3			52,7	2,4145
					13	704,8			48,4	1,8272
					14	736,4			60,5	2,0665
3	25	17	36	Healthy	15	654,7		2	55,0	1,6967
					16	695,3			56,2	1,8707
					17	569,2			51,0	1,5444
					18	551,3			51,2	1,5227
					19	626,6			57,2	2,0447
					20	614,3			55,7	1,8055

TABLE NO.7.14C(CONTD.)

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
4	24	17	35	Healthy	21	699,3		0	58,8	1,8490
					22	718,1			58,9	2,0665
					23	503,6			42,8	1,6532
					24	657,4			48,1	2,0882
					25	716,3			61,3	1,8925

TABLE NO. 7.14C

CONTROL : (SACRIFICE DAY 18 p.c.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	21	18	31	Healthy	1	939,3		0	66,3	2,2623
					2	936,4			68,3	2,4145
					3	907,2			67,3	2,3058
					4	931,8			62,2	2,3275
					5	1002,9			62,8	2,3928
					6	945,6			69,1	2,2840
2	23	18	34	Healthy	7	1089,9		2	65,3	2,4798
					8	991,3			62,5	2,6973
					9	947,1			66,0	2,4363
					10	966,5			71,1	2,3493
					11	987,4			69,7	2,3493
3	21	18	34	Healthy	12	1000,0		0	63,9	2,4798
					13	1039,1			56,7	2,6538
					14	1098,5			74,3	2,5015
					15	1062,5			72,5	2,7408
					16	1038,3			69,4	2,4145
					17	1052,1			69,4	2,4363
					18	1023,8			71,5	2,4580

TABLE NO. 7.17T

TEST : CYCLOPHOSPHAMIDE 7,5 mg/Kg ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	21,5	19	30,0	Healthy	1	1246,0	Normal	3	78,2	2,839
					2	1188,0			78,3	2,871
2	23,5	19	37,5	Healthy	3	1317,0	Normal	1	78,2	2,937
					4	1277,0	"		81,7	3,132
					5	1182,0	"		80,3	3,23
					6	1270,0	"		85,7	3,426
					7	1225,0	"		85,1	3,344
					8	1386,0	"		85,6	3,263
3	24,5	19	42,5	Healthy	9	1325,0	Normal	0	84,2	3,393
					10	1269,0	"		82,4	3,393
					11	1081,4	"		74,9	3,23
					12	1312,0	"		84,1	3,459
					13	1178,0	"		79,4	3,23
					14	1029,0	"		77,7	3,344
					15	960,0	"		74,8	3,344
					16	1249,0	"		79,5	3,328
4	23,5	19	41,0	Healthy	17	1221,0	Normal	0	80,7	3,377
					18	1265,0	"		77,2	3,067
					19	1211,0	"		79,1	3,426
					20	1185,0	"		81,9	3,524
					21	1204,1	"		78,9	3,377
					22	1287,0	"		85,5	3,687

TABLE NO. 7.17C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	25,5	19	38,5	Healthy	1	1402,0	Normal	1	90,4	3,344
					2	1517,5	"		90,6	3,557
					3	1483,0	"		93,1	3,296
					4	1511,1	"		91,8	3,328
2	22,5	19	42,5	Healthy	5	1356,9	2 fresh IUD's Normal	0	85,4	3,067
					6	1307,0	"		86,3	3,067
					7	1093,0	"		75,8	3,018
					8	1400,0	"		88,1	3,736
					9	1326,0	"		83,1	3,296
3	24,0	19	40,0		10	1321,0	Normal	0	85,5	3,426
					11	1364,0	"		88,2	3,459
					12	1338,0	"		85,1	3,344
					13	1343,0	"		80,1	3,312
					14	1314,0	"		86,3	3,051
					15	1191,0	"		71,0	2,855
					16	1329,0	"		81,4	3,23
4	24,5	19	44,0	Healthy	17	1348,0	Normal		87,6	3,426
					18	1330,0	"	0	86,9	3,312
					19	1330,5	"		89,4	3,23
					20	1399,0	"		85,9	3,442
					21	1408,0	"		88,3	3,377
					22	1129,0	"		76,2	3,002

TABLE NO. 7.19

TEST : CYCLOPHOSPHAMIDE 12,5 mg/Kg ON DAY 8½ p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	23	18	27	Healthy			No live fetuses	6		
2	24,5	18	32	Healthy			1 live small fetus no gross abnormalities	5		
3	24	18	29,5	Healthy			No live fetuses	7		

TABLE NO. 7.20T

TEST : CYCLOPHOSPHAMIDE 12,5 mg/Kg ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	26,0	14	32,0	Healthy	1	149,2	Normal	1	60,4	0,924
					2	150,2	"		73,7	1,175
					3	141,1	"		64,0	0,979
					4	148,7	"		61,3	0,892
					5	154,6	"		51,9	1,00
					6	148,8	"		71,6	1,109
2	23,5	14	29,0	Healthy	7	142,0	Normal	1	50,0	1,196
					8	128,4	"		60,0	1,022
					9	119,3	"		49,8	0,935
					10	153,2	"		56,8	0,935
					11	174,3	"		63,4	1,044
					12	123,4	"		55,2	1,00
3	26,5	14	33,0	Healthy	13	109,0	Normal	0	33,2	0,87
					14	100,6	"		30,0	0,750
					15	107,8	"		30,9	0,914
					16	138,6	"		41,4	1,088
					17	148,6	"		47,8	1,088
					18	109,7	"		36,2	1,131
					19	150,0	"		47,7	1,066
					20	97,1	"		33,6	1,088

TABLE NO. 7.20C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Head weight in mg	Brain AChE in nmol/min/mg
1	26,0	14	34,0	Healthy	1	157,1	Normal	0	63,5	0,979
					2	150,4	"		65,5	0,979
					3	161,5	"		69,4	0,946
					4	155,9	"		62,2	0,979
					5	141,8	"		61,7	1,044
					6	166,5	"		63,7	0,957
					7	169,8	"		77,7	1,218
					8	151,2	"		65,3	0,979
2	27,0	14	32,0	Healthy	9	185,7	Normal	1	67,1	1,175
					10	168,6	"		53,4	1,011
					11	176,4	"		68,6	1,153
					12	203,8	"		87,3	0,674
					13	189,2	"		66,5	1,218
					14	173,9	"		62,8	1,022
3	26,0	14	31,5	Healthy	15	155,2	Normal	0	51,1	1,011
					16	197,1	"		62,0	1,088
					17	166,0	"		52,5	1,044
					18	171,9	"		60,5	1,12
					19	184,2	"		62,0	1,109
					20	166,5	"		58,8	1,066

TABLE NO. 7.22T

TEST : CYCLOPHOSPHAMIDE 20 mg/Kg ON DAY 12 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	27,0	13	30,0	Healthy	1	117,2	Normal	1	46,6	0,805
					2	126,2	"		48,5	0,739
					3	112,1	"		45,1	0,827
					4	132,6	"		53,7	0,783
					5	130,3	"		55,6	0,848
					6	116,0	"		50,0	0,783
					7	111,8	"		52,4	0,827
2	28,0	13	31,0	Healthy	8	90,5	Normal	0	37,8	0,761
					9	119,5	"		47,1	0,848
					10	101,3	"		37,8	0,761
					11	104,4	"		39,5	0,848
					12	100,9	"		41,7	0,827
					13	104,2	"		46,4	0,870
					14	105,5	"		42,9	0,827
					15	117,2	"		40,2	0,783
3	27,0	13	31,0	Healthy	16	118,7	Normal	0	48,1	0,761
					17	113,0	"		47,6	0,761
					18	130,8	"		51,1	0,783
					19	114,4	"		45,2	0,783
					20	113,6	"		42,5	0,805
					21	105,5	"		42,9	0,805

TABLE NO. 7.22C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	27,0	13	29,0	Healthy	1	115,9	Normal	2	44,5	0,718
					2	111,0	"		41,5	0,740
					3	97,8	"		43,6	0,892
					4	106,3	"		42,0	0,805
					5	101,6	"		42,9	0,740
2	26,0	13	28,5	Healthy	6	110,0	Normal	0	45,3	0,827
					7	118,8	"		49,2	0,827
					8	119,2	"		50,9	0,892
					9	131,7	"		60,9	0,870
					10	121,4	"		51,0	0,794
3	27,0	13	31,0	Healthy	11	117,4	Normal	0	54,2	0,805
					12	140,2	"		54,4	0,794
					13	89,7	"		38,3	0,696
					14	129,8	"		55,2	0,761
					15	117,9	"		50,3	0,805
					16	116,7	"		53,9	0,870
					17	129,9	"		58,9	0,870
					18	126,0	"		55,2	0,979

TABLE NO. 7.24T

TEST : CYCLOPHOSPHAMIDE 20 mg/Kg ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Head weight in mg	Brain AChE in nmol/min/mg
1	24	12	27,5	Healthy	1	-		1	-	
					2	38,5			11,0	1,37
					3	40,6	Haemorrhagic areas in brain		13,5	1,925
					4	-			-	-
					5	-			-	-
					6	37,6			11,6	1,631
					7	-			-	-
2	27	12	29	Healthy	8	32,4	1 dead fetus	2	13,4	1,109
					9	34,2			13,7	1,142
					10	34,7			15,7	1,175
					11	24,6			12,5	1,24
					12	38,8			14,7	1,142
3	25,5	12	29	Healthy	13	38,0	1 macerated IUD	0	14,8	1,109
					14	49,6			20,4	1,142
					15	30,9	Haemorrhagic areas in brain		11,3	1,175
					16	43,8	" " "		16,1	1,109
					17	51,0			19,2	1,109
					18	54,6			21,4	1,24
					19	46,9			18,2	1,109
					20	39,2			12,0	1,272

TABLE NO. 7.24T (Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Head weight in mg	Brain AChE in nmol/min/mg
4	25	12	28,5	Healthy	21	40,4		1	15,2	1,109
					22	45,2			15,4	1,175
					23	44,3			14,3	1,044
					24	50,5	Haemorrhagic area in brain		20,7	1,207
					25	49,3			19,0	1,175
					26	50,5			19,9	1,305

TABLE NO. 7.24C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	26	12	29	Healthy	1	71,3		1	27,8	1,109
					2	60,6			19,4	1,24
					3	58,2			19,9	1,305
					4	64,2			25,3	1,175
					5	55,1			20,8	1,175
					6	82,9			26,5	1,109
					7	65,6			24,4	1,175
2	25,5	12	29	Healthy	8	81,2		0	23,1	1,175
					9	59,9			20,4	1,044
					10	66,1			25,4	1,175
					11	71,4			26,6	1,109
					12	91,8			29,1	1,077
					13	63,9			19,8	1,175
3	23	12	31,5	Healthy	14	71,9		0	26,5	1,109
					15	67,0			25,8	0,979
					16	65,5			23,8	1,044
					17	94,5			33,2	1,109
					18	79,2			29,1	0,979
					19	84,5			31,3	1,044
					20	77,4			34,0	1,044

TABLE NO. 7.26T

TEST : CYCLOPHOSPHAMIDE 20 mg/Kg on DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Head weight in mg	Brain AChE in nmol/min/mg
1	24	12	28	Healthy	1	49,6		0	12,4	1,305
					2	48,1			9,3	1,240
					3	45,4			11,4	1,305
					4	35,3			5,7	-
					5	50,5			11,3	1,109
					6	49,2			14,8	1,109
					7	49,4			8,0	1,305
2	24,5	12	28,5	Healthy	8	-	(Damaged)	0	-	-
					9	55,2			11,4	1,305
					10	41,6	Haemorrhagic areas in brain		8,2	1,436
					11	57,6	" " "		14,0	1,240
					12	56,6			22,9	1,175
					13	43,0			9,1	1,272
					14	41,8			13,1	1,240
3	26	12	30	Healthy			1 dead haemmorha-gic fetus			
					15	42,3		1	7,4	1,142
					16	53,4	Haemorrhagic areas in brain		12,0	1,175
					17	51,2			19,8	1,240
					18	50,0			14,9	1,240
					19	52,0			13,3	1,240
					20	57,6			12,8	1,272

TABLE NO. 7.26C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Head weight in mg	Brain AChE in nmol/min/mg
1	23	12	27,5	Healthy	1	61,9		0	13,9	1,109
					2	63,2			18,3	1,109
					3	78,7			31,1	1,109
					4	61,6			20,5	1,044
					5	81,5			26,3	1,109
					6	70,9			17,0	1,109
					7	60,6			18,3	1,175
2	24,5	12	29	Healthy	8	63,1		0	13,9	1,240
					9	73,0			23,0	1,109
					10	76,1			19,1	1,175
					11	60,2			13,9	1,207
					12	56,1			14,7	1,175
					13	69,7			19,1	1,175
3	25	12	29	Healthy	14	77,0		2	23,5	0,848
					15	65,3			12,6	1,272
					16	70,4			21,2	-
					17	99,0			25,0	0,946
					18	66,2			21,7	1,175
4	27,5	12	31,5	Healthy			1 dead fetus			
					19	59,0		1	14,7	1,272
					20	60,1			14,8	1,109
					21	73,5			17,6	1,175
					22	64,7			17,0	1,175

TABLE NO. 7.33T TEST : CYCLOPHOSPHAMIDE 15mg/kg ADMINISTERED ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain * ChAT activity as d.p.m.
1	23,5	18	31	Healthy	1	991,6		4	64,5	231
					2	976,3			62,4	505
					3	923,1			60,3	342
					4	894,9			62,2	560
2	24,5	18	34	Healthy			2 IUD's ± 11-12 days			
					5	816,8		0	58,7	556
					6	823,8			60,0	604
					7	883,7			53,6	668
					8	744,7			55,9	Spoilt
					9	846,7			59,7	913
3	25	18	37	Healthy	10	820,9		1	44,0	371
					11	783,3			58,3	805
					12	843,7			59,7	752
					13	845,8			56,1	815
					14	763,3			57,3	552
					15	839,2			48,9	555
					16	788,7			56,5	642
					17	821,8			55,3	546
4	23,5	18	33	Healthy			1 IUD ± 15 days			
					18	775,0	Exophthalmos	2	57,1	364
					19	774,1			56,5	581

* Actual d.p.m. (blank value subtracted)

TABLE NO.7.33T (Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain * ChAT activity as d.p.m
4	23,5	18	33	Healthy	20	764,1		2	56,7	455
					21	722,1			49,3	703
					22	768,8			54,3	744

* Actual d.p.m. (blank value subtracted)

TABLE NO.7.33C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain * ChAT activity as d.p.m
1	22	18	34	Healthy	1	1145,5		0	73,8	647
					2	1108,0			75,4	630
					3	1114,6			69,2	718
					4	1151,6			70,7	326
					5	1126,4			66,4	799
					6	1094,5			74,3	218
2	23	18	37	Healthy	7	1055,6		0	72,1	702
					8	1048,7			65,5	813
					9	1103,7			74,5	879
					10	1071,0			72,8	580
					11	1085,9			66,0	749
					12	1047,7			59,7	1124
					13	983,8			64,5	693
3	24,5	18	37	Healthy	14	1073,3		0	66,7	877
					15	1023,7			65,6	613
					16	1006,4			68,5	875
					17	1054,6			68,8	752
					18	966,8			66,8	594
					19	997,6			68,1	674
					20	1004,3			67,8	947
					21	1045,5			69,8	1054

* Actual d.p.m. (blank value subtracted)

TABLE NO. 7.35T

TEST : CYCLOPHOSPHAMIDE 20mg/kg ON DAY 10 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Head weight in mg	Brain ChAT activity as d.p.m.*
1	24,0	12	25,5	Healthy	1	48,0	1 IUD - large haemorrhagic area over entire brain	0	17,8	674
					2	48,5			17,4	592
					3	41,4			12,7	584
					4	46,9			17,9	843
					5	43,9			16,2	691
					6	38,8			12,5	639
2	24,5	12	26,5	Healthy	7	47,6		2	16,2	643
					8	60,5			20,9	700
					9	53,3	Small cerebral haemorrhagic area		17,3	683
					10	54,4			21,0	709
					11	57,4			26,3	590
					12	48,9			14,9	613
					13	54,2			17,7	623
3	26,0	12	27,5	Healthy	14	47,6	1 IUD	1	18,5	696
					15	55,4	Cerebral haemorrhagic areas		19,8	616
					16	57,8			23,7	642
					17	58,7			21,7	701

* Total d.p.m. (Blank value not subtracted)

TABLE NO. 7.35T (Contd.)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Head weight in mg	Brain ChAT activity as d.p.m.*
3	26,0	12	27,5	Healthy	18	59,7	Blood stained liquor		22,1	601
					19	60,1			30,4	920
					20	54,7	Blood stained liquor		20,9	740

* Total d.p.m. (blank value not subtracted)

TABLE NO. 7.35C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain ChAT activity as d.p.m.*
1	23,5	12	24,5	Healthy	1	58,3		0	26,5	702
					2	62,3			18,8	543
					3	61,5			24,7	603
					4	63,9			30,0	588
					5	55,5			26,2	700
					6	56,7			21,0	667
2	24,0	12	25,5	Healthy	7	70,0		0	24,4	730
					8	68,1			21,0	635
					9	70,6			24,1	601
					10	68,3			26,0	709
					11	72,9			23,8	748
					12	68,1			27,8	846
3	24,0	12	25	Healthy	13	64,8		1	24,2	793
					14	57,8			24,7	795
					15	66,0			28,5	853
					16	70,8			22,9	804
4	23,5	12	25	Healthy	17	75,8		0	31,9	803
					18	59,5			24,4	831
					19	74,9			34,7	880
					20	89,4			38,4	732
					21		Spoilt			
					22		Spoilt			

* Total d.p.m. (blank value not subtracted)

TABLE NO. 8.1.

TEST : SODIUM VALPROATE, 400mg/kg SUBCUTANEOUSLY ON DAYS 8 AND 9 p.c.

(2h MATING)

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	19	Healthy		1 macerated, small IUD	
				2 fresh IUD's, macroscopically normal	
			1	Exencephaly with blood stained hydramnios	-
			2	Normal	
			3	"	
			4	"	
			5	"	
2	19	Healthy	6	Exencephaly with exophthalmos	2
			7	" " "	
			8	Normal	
			9	"	
3	19	Healthy		1 small IUD with exencephaly	
			10	Normal	
			11	"	
			12	"	
			13	"	
			14	"	
			15	"	
			16	"	

TABLE NO. 8.1. (CONTD) TEST

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
4	19	Healthy	17	1 fresh IUD	
			18	Exencephaly with exophthalmos	
			19	" " "	
			20	Normal	
			21	"	

TABLE NO. 8.2. SODIUM VALPROATE, 400mg/kg ON DAYS 10, 11 AND 12 p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	19	Healthy	1 2 3 4 5 6 7		0
2	19	Healthy	8 9 10 11 12	1 macerated IUD	0
3	19	Healthy	13 14 15 16 17 18 19 20		0

TABLE NO. 8.3. TEST : SODIUM VALPROATE, 800mg/kg ON DAY 10, 400mg/kg ON DAYS 11 AND 12 p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	-	Died after initial dose		-	
2	19	Transient drowsiness and ataxia after initial dose		No live fetuses	7
3	-	Died after initial dose		-	
4	19	Transient drowsiness and ataxia	1	Normal	6

TABLE NO. 8.4. TEST : SODIUM VALPROATE, 520mg/kg s.c. ON DAYS 8 AND 9 p.c.

MATERNAL PARAMETERS			FETAL PARAMETERS		
MOUSE NO.	DAY OF SACRIFICE	PHYSICAL STATE	FETUS NO.	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	19	Healthy	1	4 macerated, tiny IUD's with hydramnios Exencephaly, exophthalmos, blood stained hydramnios	0
			2	" " "	
			3	" " "	
			4	" " "	
			5	" " "	
2	19	Healthy	6	2 macerated, tiny IUD's Exencephaly with exophthalmos	2
			7	" " "	
			8	" " "	
			9	Normal	
			10	"	

TABLE NO. 8.6T SODIUM VALPROATE, 400mg/kg, ADMINISTERED ON DAYS 16 AND 17 p.c.

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	22,0	18	34,0	Healthy	1	1080,2	Normal	0	71,3	3,05
					2	1101,3	"		68,0	2,85
					3	1004,9	"		69,5	3,20
					4	966,9	"		72,6	2,94
					5	976,8	"		69,9	3,03
					6	1039,2	"		70,0	2,94
					7	1008,4	"		68,8	2,89
2	23,0	18	36,5	Healthy	8	1039,4	1 IUD ± term. No abnormalities Normal	0	70,1	3,10
					9	958,4	"		64,6	3,01
					10	943,1	"		66,2	2,97
					11	1049,7	"		65,5	3,07
					12	1030,5	"		68,9	2,98
					13	1048,6	"		68,5	2,85
3	23,0	18	37,0	Healthy	14	986,8	Normal	1	65,6	2,97
					15	989,4	"		64,3	2,84
					16	1016,9	"		69,2	2,89
					17	889,4	"		67,9	2,77
					18	954,3	"		64,0	2,90
					19	998,2	"		67,0	2,81

TABLE NO. 8.6C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	21,5	18	34,5	Healthy	1	989,2	Normal	1	68,4	2,84
					2	1016,9	"		69,9	2,98
					3	954,3	"		65,4	3,03
					4	998,2	"		64,2	2,78
					5	1049,7			70,8	3,10
					6	1048,6	"		66,2	2,82
2	22,5	18	37,0	Healthy	7	1042,3	Normal	1	70,4	3,08
					8	942,6	"		69,1	2,79
					9	1054,2	"		74,3	3,10
					10	983,1	"		62,4	2,78
					11	970,0	"		65,3	2,92
					12	998,1	"		68,1	2,80
					13	1042,0	"		70,2	2,65
3	23,0	18	36,5	Healthy	14	958,2	1 IUD ± term. No abnormality. Normal	0	64,3	2,95
					15	981,4	"		62,1	2,94
					16	973,4	"		65,4	2,83
					17	1082,3	"		70,3	3,10
					18	1010,4	"		74,1	2,74
					19	992,4	"		64,2	2,84
					20	1042,3			69,9	2,95

TABLE NO. 8.8T

TEST : SODIUM VALPROATE, 400mg/kg, ADMINISTERED ON DAYS 8 AND 9 p.c.
(FIRST EXPERIMENT)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	24	19	37,5	Healthy	1	1338	Normal	1	90,8	3,49
					2	1243	"		84,2	3,46
					3	1330	"		86,3	3,51
					4	1265	"		88,5	3,31
					5	1329	"		88,0	3,31
2	22,5	19	34	Healthy	6	1021	1 macerated IUD Exomphalos	6	72,8	2,94
3	22	19	35	Healthy	7	1395	Normal	2	85,5	3,16
					8	1352	"		84,0	3,39
					9	1195	"		79,4	3,03
					10	1362	"		86,2	3,23
4	23	19	35	Healthy	11	1289	Exomphalos	2	81,4	3,03
					12	1255	Normal		79,2	2,94
					13	1351	"		84,8	3,25
					14	1291	"		84,8	3,16
5	22,5	19	38,5	Healthy	15	1247	Normal	0	87,7	2,94
					16	1191	"		83,2	2,85
					17	1212	"		87,4	2,97
					18	1150	"		88,2	3,08
					19	1170	"		86,0	3,10
					20	1201	"		86,8	3,07

TABLE NO. 8.8C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	21	19	36	Healthy	1	1362	Normal	0	85,4	3,20
					2	1328	"		84,3	2,87
					3	1371	"		84,6	2,98
					4	1187	"		86,9	3,36
					5	1289	"		85,3	3,20
					6	1277	"		86,2	3,20
2	22,5	11	37	Healthy	7	1380	Normal	2	105,0	2,87
					8	1425	"		94,9	3,10
					9	1379	"		96,3	3,03
					10	1328	"		94,9	2,84
					11	1458	"		98,3	2,97
3	21	11	33	Healthy	12	1332	Normal	0	85,1	3,12
					13	1418	"		88,7	3,20
					14	1311	"		88,8	3,15
					15	1360	"		86,7	3,20
					16	1399	"		90,2	3,21
					17	1414	"		93,0	3,20
4	21	11	33	Healthy	18	1423	Exomphalos	0	92,2	2,94
					19	1480	Normal		95,8	2,84
					20	1366	"		88,3	2,87
					21	1306	"		95,0	2,98

TABLE NO. 8.1OT

TEST : SODIUM VALPROATE, 400mg/kg, ADMINISTERED ON DAYS 8 AND 9 p.c.
(SECOND EXPERIMENT)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	21,5	19	33	Healthy	1	1230	Normal	4	78,2	3,39
					2	1180	"		73,1	3,13
					3	1120	"		66,4	3,64
2	25	19	39	Healthy	4	1390	Normal	0	82,8	3,23
					5	1440	"		84,6	3,36
					6	920,0	Runt		57,6	2,84
					7	840,0	"		67,6	3,07
					8	1410	Exomphalos		85,0	3,16
3	23,5	19	35	Healthy			1 small IUD with early exencephaly, strangulated exomphalos			
					9	1280	Normal	1	83,8	3,21
					10	1260	"		71,6	3,29
					11	1400	"		88,2	3,44
					12	1190	Exencephaly		74,1	2,15
4	24	19	36	Healthy			1 fresh IUD with exencephaly			
					13	1001	Normal		16,6	-
					14	1250	"		83,4	3,23
					15	1200	"		82,2	3,20
					16	1200	"		69,6	3,16

TABLE NO. 8.1OT (Contd.)

TEST : SODIUM VALPROATE, 4OOmg/kg, ADMINISTERED ON DAYS 8 and 9 p.c.
(SECOND EXPERIMENT)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
5	22	19	39	Healthy			1 fresh IUD			
					17	1260	Normal		71,9	3,13
					18	1230	"		76,2	3,16
					19	1230	"		80,1	3,10
					20	1260	"		68,3	3,33
					21	1320	"		80,6	3,33
					22	1230	"		76,0	3,20

TABLE NO. 8.10C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	22	19	34	Healthy	1	1400	Normal	0	83,0	3,39
					2	1370	"		80,9	3,16
					3	1350	"		84,3	3,44
2	24,5	19	43	Healthy	4	1430	Normal	0	90,5	3,52
					5	1410	Exomphalos		91,4	3,46
					6	1380			81,5	3,13
					7	1350			85,5	3,34
					8	1260			81,6	3,36
					9	1220			80,7	3,26
					10	1390			87,2	3,25
3	22	19	41,5	Healthy	11	1210	1 small IUD	0	71,0	3,15
					12	1210			73,5	3,26
					13	1230			75,0	3,42
					14	1360			86,4	3,60
					15	1250			75,5	3,00
					16	1370			82,5	3,52
					17	1380			85,6	3,60

TABLE NO. 8.12T

TEST : SODIUM VALPROATE, 440mg/kg s.c. ON DAYS 8 AND 9 p.c.

(FIRST EXPERIMENT)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacrifice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorptions	Brain weight in mg	Brain AChE in nmol/min/mg
1	25	19	41,5	Healthy			1 IUD with exencephaly.			
					1	1340	Normal	0	87,0	2,71
					2	1300	"		82,5	2,77
					3	1296	"		84,0	3,00
					4	1190	"		85,2	2,97
					5	1250	"		83,3	3,13
					6	1180	"		77,3	2,97
2	23,5	19	41	Healthy			1 IUD: exencephaly and exophthalmos			
					7	1150	Normal	0	82,5	3,34
					8	1260	"		85,0	3,51
					9	1260	"		76,4	3,13
					10	1130	"		82,2	3,49
					11	1210	"		79,2	3,20
					12	1240	"		82,6	3,12
					13	1320	"		80,4	3,47
					14	1180	"		65,7	3,62
3	22	19	37	Healthy	15	1110	Exencephaly, exophthalmos	0	59,3	3,34
					16	1050	" "		27,0	4,73
					17	1200	Exencephaly, exomphalos		23,0	too small for assay

TABLE NO.8.12T(CONTD)

TEST

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
3	22	19	37	Healthy	18	1190	Normal	0	75,2	3,42
					19	1120	"		70,1	3,44
					20	1330	"		80,4	3,52
4	23	19	37	Healthy	21	1020	Exencephaly, ex-ophthalmos	2	22,7	Sample too small
					22	1160	Exencephaly		39,1	6,07

TABLE NO. 8.12C

CONTROL

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	23,5	19	36	Healthy	1	1430	Normal	0	89,4	3,59
					2	1350	"		84,6	3,33
					3	1400	"		90,0	3,59
					4	1270	"		82,5	3,21
					5	1230	"		81,7	3,39
					6	1010	"		82,6	3,42
2	23,5	19	37	Healthy	7	1370	Normal	0	83,4	3,26
					8	1280	"		79,4	3,39
					9	1310	"		85,8	3,26
					10	1270	"		88,7	3,26
					11	1250	"		79,1	2,94
					12	1290	"		81,7	3,20
					13	1370	"		87,8	3,36
3	21	19	39,5	Healthy	14	1330	Normal	0	82,9	3,85
					15	1200	"		75,8	3,49
					16	1320	"		80,2	3,60
					17	1390	"		80,4	3,82
					18	1210	"		76,5	3,59
					19	1250	"		81,1	3,75
					20	1210	"		87,8	4,01

TABLE NO. 8.14T

TEST : SODIUM VALPROATE, 440mg/kg, ADMINISTERED ON DAYS 8 AND 9 p.c.
(SECOND EXPERIMENT)

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	25,5	19	40	Healthy	1	980	Exencephaly with exophthalmos	1	27,8	3,59
					2	1080	" "		19,9	-
					3	1160	Normal		77,8	3,33
					4	1200	"		80,8	3,00
					5	1190	"		82,5	3,39
					6	1170	"		79,4	3,26
					7	1080	"		67,8	3,39
2	22,5	19	33,5	Healthy	8	1380	Normal	2	86,6	3,20
					9	1300	"		85,5	3,39
					10	1270	"		82,0	3,26
3	27	19	41	Healthy	11	1070	Normal	1	75,6	3,33
					12	1300	"		84,4	3,29
					13	1290	"		84,1	3,23
					14	1190	"		82,2	3,16
					15	1200	"		81,1	3,46
					16	1170	"		76,8	3,43
					17	1290	"		79,7	3,10
4	24	19	39	Healthy	18	1240	Normal	2	79,8	3,05
					19	1320	"		85,8	3,46
					20	1260	"		88,4	3,59
					21	1080	Exencephaly, exoph-thalmos.		20,6	4,69
					22	1260	Exencephaly		19,8	5,92

TABLE NO. 8.14C

CONTROLS

MATERNAL PARAMETERS					FETAL PARAMETERS					
Mouse no.	Weight in grams on day of dosing	Day of sacri-fice	Weight in grams on day of sacrifice	Physical state	Fetus no.	Weight in mg	Obvious external abnormality or death	Resorp-tions	Brain weight in mg	Brain AChE in nmol/min/mg
1	25	19	40	Healthy	1	1280	Normal	1	87,0	3,49
					2	1370	"		85,9	3,52
					3	1200	"		82,8	3,51
					4	1240	"		86,2	3,39
					5	1420	"		85,4	3,64
					6	1180	"		85,2	3,60
2	24	19	40	Healthy	7	1330	Normal		84,7	3,28
					8	1320	"		84,6	3,29
					9	1190	"		82,2	3,29
					10	1350	"		83,4	3,07
					11	1260	"		83,3	3,26
					12	1320	"		82,7	3,29
					13	1310	"		86,8	3,46
3	24	19	37	Healthy	14	1510	Normal	1	93,1	3,52
					15	1430	"		91,3	3,36
					16	1460	"		94,7	3,29
					17	1530	"		88,6	3,29
					18	1170	"		83,0	3,16
					19	1080	"		84,5	3,57
					20	1020	"		73,7	3,26
					21	1240	"		79,1	3,13

TABLE NO. 9.2. VITAMIN A, 5 000 IU, ADMINISTERED 60h POST COPULATION (SACRIFICE DAY 18 p.c.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	40	Healthy			1 IUD ± 12 days	0
			1	1041,1	Nil	
			2	1054,2	"	
			3	1006,1	"	
			4	1150,8	"	
			5	1066,8	"	
			6	1057,2	"	
			7	1099,1	"	
2	41	Healthy			1 IUD ± 17 days	0
			1	1041,1	Nil	
			2	1054,2	"	
			3	1006,1	"	
			4	1150,8	"	
			5	1066,9	"	
			6	1057,2	"	
			7	1099,1	"	
			8	969,0	"	
3	35,5	Healthy	1	1194,9	Nil	2
			2	1112,0	"	
			3	1127,7	"	
			4	1254,6	"	

TABLE NO. 9.2. (CONTD.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
4	40	Healthy	1	548,7	Runt - Nil	0
			2	1056,6	Nil	
			3	882,2	"	
			4	926,9	"	
			5	1043,8	"	
			6	975,0	"	
			7	1005,9	"	
			8	1002,6	"	
			9	1026,8	"	
5	41,5	Healthy	1	1101,7	Nil	0
			2	1030,8	"	
			3	1096,8	"	
			4	1124,4	"	
			5	1107,6	"	
			6	1045,3	"	
			7	1092,9	"	
			8	962,8		
6	37	Healthy			2 IUD's ± 12 days	0
			1	1159,9	Nil	
			2	1031,1	"	
			3	1278,5	"	

TABLE NO. 9.2. (CONTD.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
6	37	Healthy	4 5 6	1151,4 1108,3 1255,2	Nil " "	0

TABLE NO. 9.3. VITAMIN A, 10 000 IU, ADMINISTERED 60h POST COPULATION (SACRIFICE DAY 18 p.c.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	38	Healthy	1	871,1	Exencephaly, blood stained hydromnios	1
			2	724,0	" " " "	
			3	785,9	Pug shaped face. Exomphalos	
			4	952,4	Pug shaped face	
			5	1005,1	" " "	
			6	944,8	" " "	
2	32	Healthy			One IUD ± 13 days	
			7	1032,9		0
			8	1057,2		
			9	1142,0		
3	41	Healthy	1	1003,9	Normal	0
			2	1018,8	"	
			3	1097,0	"	
			4	981,1	"	
			5	1039,2	"	
			6	1129,4	"	
			7	1139,8	"	
			8	1050,6	"	

TABLE NO. 9.3.(CONTD.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
4	38,5	Healthy			1 IUD ± 17 days	
			1	1201,3	Nil	0
			2	996,1	"	
			3	1108,8	"	
			4	1102,6	"	
			5	1039,1	"	
			6	1045,6	"	
5	41	Healthy			1 IUD ± 17 days	
			1	963,4	Nil	0
			2	1075,9	"	
			3	1022,0	"	
			4	1003,4	"	
			5	1147,9	"	
			6	975,7	"	
			7	1115,3	"	
6	41,5	Healthy	1	1080,4	Nil	0
			2	1058,9	"	
			3	1040,2	"	
			4	1048,1	"	
			5	1097,1	"	
			6	1062,0	"	
			7	985,9	"	
			8	1078,4	"	

TABLE NO. 9.4. VITAMIN A, 15 000 IU, ADMINISTERED 60h POST COPULATION (SACRIFICE DAY 18 p.c.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	35	Healthy			One IUD ± 14 days. No abnormality	0
					One IUD ± term " "	
			1	837,9	Prominent eyes. Small for age	
			2	868,0	" " " " "	
			3	685,0	" " " " "	
			4	782,0	" " " " "	
			5	934,3	" " " " "	
			6	930,0	Prominent eyes. Small for age and exomphalos	
2	34,5	Healthy	7	926,2	Exomphalos, kinked tail	1
			8	682,8		
			9	993,5		
			10	1065,5		
			11	1082,9		
			12	974,6		
3	32,5	Healthy			One IUD ± 13 days	2
			13	814,4	Exencephaly, exophthalmos, blood stained hydramnios	
			14	1008,5		
			15	1050,6		
			16	1010,5		

TABLE NO. 9.4. (CONTD)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
4	34	Healthy	17	1080,0	Small for age	1
			18	990,0	" " "	
			19	1002,0	" " "	
			20	970,0	" " "	
			21	720,0	Exencephaly, anophthalmia	
			22	820,0	Exencephaly	
5	37	Healthy	23	988,6	Exencephaly, blood stained hydramnios	1
			24	1002,2	Kinked tail. Pug shaped face	
			25	953,9	" " " " "	
			26	1041,2	" " " " "	
			27	892,9	" " " " "	

TABLE NO. 9.5. VITAMIN A, 30 000 IU, ADMINISTERED 60h POST COPULATION (SACRIFICE DAY 18 p.c.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	32	Scrawny, hair thinning	1	949,7	Small for age	5 (± 3mm) necrotic fetal tissue with ± 5mm placen tae.
2	29,5	Scrawny, hair thinning	2	976,7	Small for age	4 (as above)
3	28	Scrawny, hair thinning	4 5	778,0 831,7	One ± 17 day IUD with blood stained hydramnios, exencephaly and exophthalmos Small for age Small for age	5 (as above)
4	25	Scrawny, hair thinning				6 (as above)
5	27	Scrawny, hair thinning			One ± 17 day IUD with blood stained hydramnios, exencephaly, microphthalmia and 4 hind limbs	8 (as above)

TABLE NO. 9.5. (CONTD.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
6	28	Scrawny, hair thinning			One ± 13 day macerated IUD.	
			6	788,5	Exencephaly, exophthalmos of one eye and anophthalmia of the other.	3 (as above)
			7	861,1	Exomphalos	
			8	933,9	Small for age	
			9	741,8	Small for age	
7	30	Scrawny, hair thinning	10	888,5	Exencephaly, anophthalmia of one eye and exophthalmos of the other eye.	5 (as above)
			11	915,7	Exencephaly, exophthalmos	
			12	798,5	Small for age	
8	31	Scrawny, hair thinning	13	565,4	Small for age	2
			14	656,7	Small for age	
			15	830,0	Small for age	
			16	796,2	Small for age	
			17	851,0	Small for age	
			18	782,0	Exomphalos and herniation of liver	

TABLE NO. 9.6.

CONTROLS - (SACRIFICE DAY 18 p.c.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	33,5	Healthy	1	817,9	Nil	O
			2	1059,8	"	
			3	1057,4	"	
			4	1035,7	"	
			5	1031,2	"	
			6	1127,5	"	
			7	983,6	"	
2	34	Healthy	8	1140,5	Nil	O
			9	1027,0	"	
			10	1126,0	"	
			11	1129,4	"	
			12	1215,3	"	
			13	637,4	Small	
			14	1057,3		
3	31,4	Healthy	15	1069,3	Nil	O
			16	962,3	"	
			17	993,9	"	
			18	962,9	"	
			19	991,4	"	
			20	960,4	"	

TABLE NO. 9.6.(CONTD.)

CONTROLS

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
4	34,8	Healthy	21	1057,9	Nil	0
			22	1046,4	"	
			23	1217,6	"	
			24	1081,3	"	
			25	1075,1	"	
			26	998,0	"	

TABLE NO. 9.7.

CONTROLS - (SACRIFICE DAY 18 p.c.)

MATERNAL PARAMETERS			FETAL PARAMETERS			
MOUSE NO.	WEIGHT ON DAY OF SACRIFICE (g)	PHYSICAL STATE	FETUS NO.	FETAL WEIGHT (mg)	OBVIOUS EXTERNAL ABNORMALITY OR DEATH	RESORPTIONS
1	29	Healthy	1	1136,0	Nil	2
			2	1041,2	"	
			3	1122,1	"	
			4	1184,4	"	
2	35	Healthy	5	1196,3	Nil	0
			6	1105,6	"	
			7	951,8	"	
			8	1044,8	"	
			9	1174,5	"	
			10	1113,1	"	
			11	1089,5	"	
3	30,5	Healthy	12	1029,9	Nil	0
			13	1092,9	"	
			14	1043,7	"	
			15	1040,3	"	
			16	865,6	"	
			17	1014,7	"	
4	28,8	Healthy			1 IUD	1
			18	1151,1	Nil	
			19	914,0	"	
			20	1010,7	"	
			21	1132,4	Thigh absent on right hind limb	

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